REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
		Final 9/1/91 to 8/31/94

4. TITLE AND SUBTITLE
Cofactor-Assisted Antibody Catalysis

5. FUNDING NUMBERS
N00014-93-MP-24023

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Office of Naval Research
800 N. Quincy St.
Arlington, VA 22217-5000

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

This report describes progress made toward the development of cofactor-assisted catalytic antibodies. The development of facile immunoassays for screening large numbers of potential catalysts directly for catalytic activity and their application to libraries of RNA receptors are also discussed.

14. SUBJECT TERMS	15. NUMBER OF PAGES
catalysis, antibodies, RNA, screening	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	UL

FINAL REPORT

GRANT #: N00014-93-MP-24023 R & T Code: 441u001

PRINCIPAL INVESTIGATOR: Donald Hilvert

INSTITUTION: The Scripps Research Institute

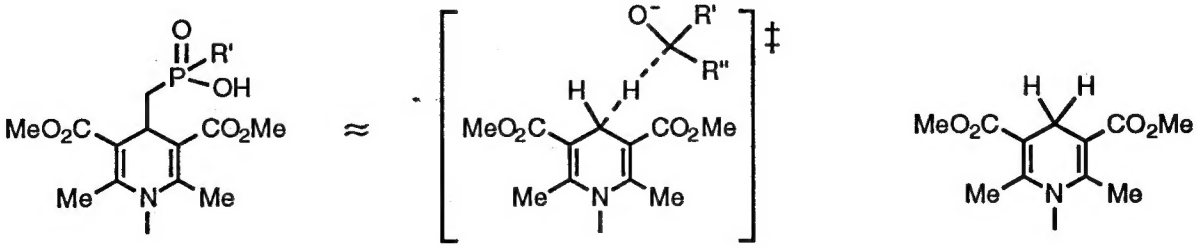
GRANT TITLE: Cofactor-Assisted Antibody Catalysis

AWARD PERIOD: 1 September 1991 - 31 August 1994

OBJECTIVE: To extend the repertoire of reactions catalyzed by antibodies through the use of chemical cofactors.

ACCOMPLISHMENTS: In this program we focused our efforts on the development of tailored antibody catalysts for redox and hydrolytic reactions promoted by nicotinamides, arylseleninic acids, *o*-iodosobenzoates, and metal ions, respectively. Progress has been made in each area as outlined below. In addition, we developed a facile immunoassay for screening large numbers of molecules directly for catalytic activity and used it to assay libraries of RNA receptors that bind a transition state analog.

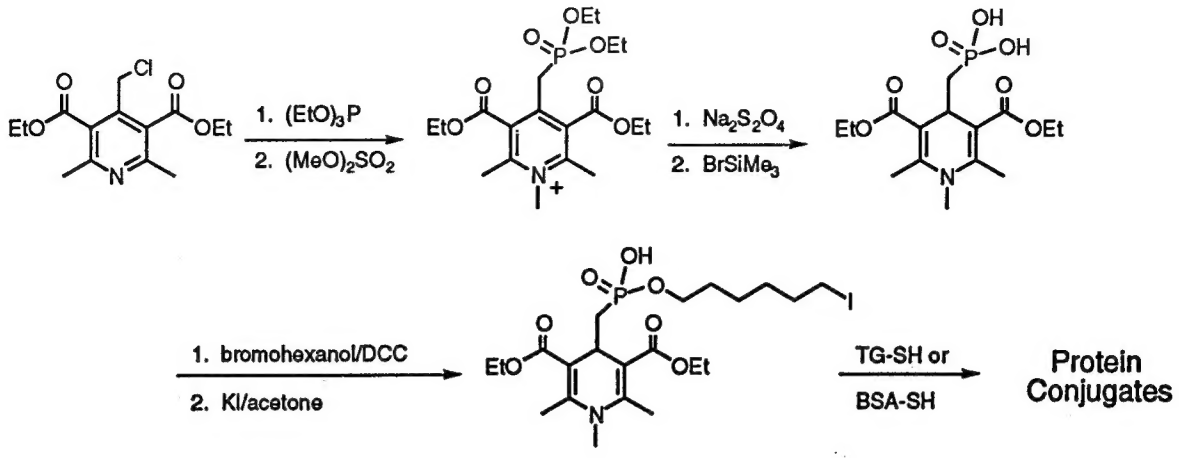
Nicotinamide cofactors. To create antibodies that utilize nicotinamide-like cofactors we designed and synthesized the Hantzsch ester **1** which mimics the presumed transition state for the reduction of aldehydes (**2**). The Hantzsch esters are attractive alternatives to nicotinamides because of their ease of synthesis and high reactivity in model systems.[1] Compound **1** was prepared and coupled to carrier proteins as shown in Scheme 1. The thyroglobulin conjugate was highly immunogenic and monoclonal antibodies were prepared by standard methods. Seventeen antibodies that exhibit high affinity binding to **1** have been selected for further study. They have been subcloned and are currently being propagated in mouse ascites. Promising results have been obtained in preliminary kinetic studies of the first seven purified antibodies: significant antibody-dependent rate accelerations have been observed spectroscopically for the reduction of propionaldehyde by the dihydropyridine derivative **3** with several clones. The remaining antibodies will be screened for their ability to speed this reaction, and the best catalysts will be characterized in detail.



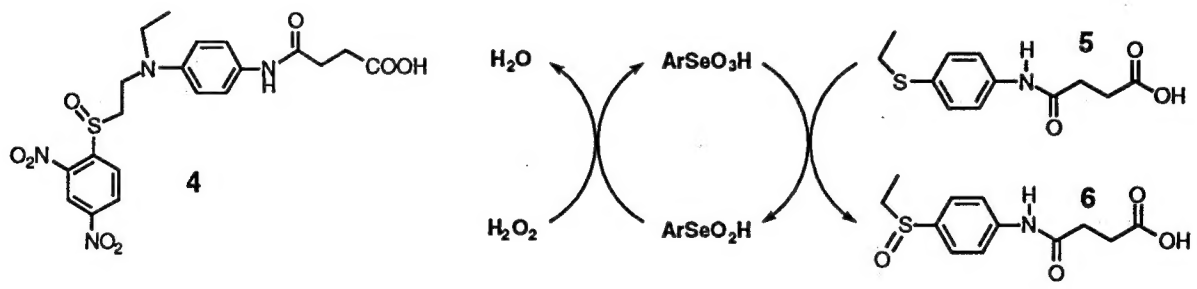
1 **2** **3**

A-1

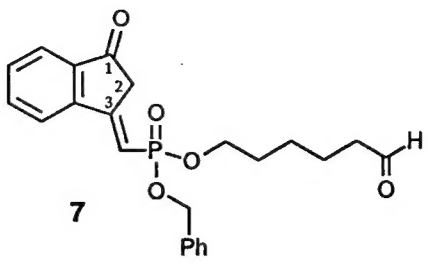
Scheme 1



Arylseleninic acid-dependent antibodies. Dinitrobenzeneseleninic acid catalyzes the oxidation of sulfides by hydrogen peroxide to sulfoxides and sulfones under heterogeneous conditions.[2] We have generated antibodies against hapten **4** which resembles the putative transition state for attack of an ethyl aryl sulfide on an aryl perseleneninic acid. Nine monoclonal antibodies were purified and tested for their ability to catalyze the oxidation of compound **5** to **6**. Kinetic experiments were carried out in aqueous buffer (pH 5.0) at 25 °C and monitored by HPLC. Antibodies 16H9 and 35G8 were found to accelerate the target reaction. For 16H9, the kinetic parameters $k_{cat} = 0.0138$ min^{-1} and $K_m = 90$ μM were determined. By way of comparison, the pseudo-first order rate constant for the seleninic acid-catalyzed reaction in the absence of protein is 3.8×10^{-5} min^{-1} at 10 mM H_2O_2 and 25 μM 2,4-dinitrobenzeneseleninic acid. The minimum rate acceleration attributable to the antibody binding site is thus about 400-fold. Detailed characterization of these catalysts is in progress.



***o*-Iodosobenzoates.** Hapten **7** was previously synthesized to create antibodies that will utilize *o*-iodosobenzoates to catalyze the hydrolysis of esters and phosphate esters.[3,4] Several high affinity antibodies that recognize this hapten were purified to homogeneity and tested for catalytic activity, but no active catalysts were found. The small sample size was perhaps limiting in this case and it would be worthwhile repeating the fusion to obtain a larger number of antibodies to test. Alternative haptens, such as **8**, which should be easier to assemble than **7**, should also be investigated.



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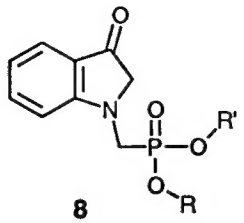

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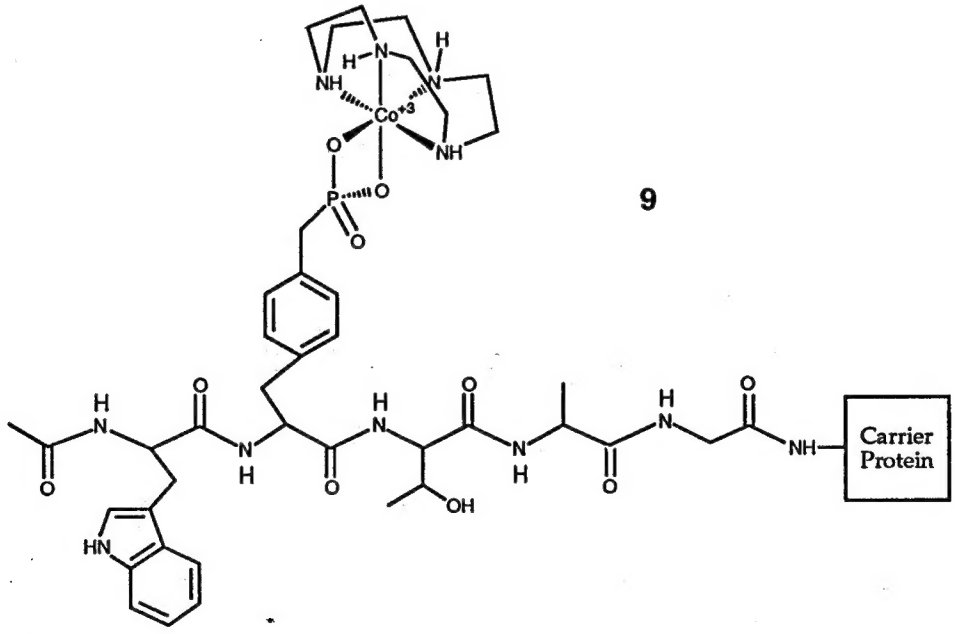
Metal dependent hydrolyses. Many naturally-occurring phosphatases contain metal ions. To generate antibodies that can similarly exploit metal-ion catalysis[5] we prepared **9**, the Co^{III}(cyclen) complex of a pentapeptide containing a phosphonotyrosine derivative. In the first set of experiments, we attempted to prepare hapten **9** by incubating the peptide (conjugated to a carrier protein) in the presence of $Co^{III}(cyclen)(H_2O)_2$. However, the purified antibodies that were raised against this hapten displayed neither catalysis nor metal-dependent peptide recognition, possibly because a stable bidentate complex between the Co^{III}(cyclen) and the phosphonotyrosine had failed to form under our reaction conditions. We have therefore modified our protocol for preparing **9**. We now incubate the peptide in the presence of a ten-fold excess of cyclen and Co(II) perchlorate and include PbO_2 to oxidize the phosphonotyrosine-Co^{II} complex in situ.[6] NMR studies on the resulting complex are consistent with the desired structure. Moreover, mice immunized with the newly prepared hapten show higher serum titers for **9** than for the metal-free peptide. These mice will be used for the preparation of monoclonal antibodies in the near future.



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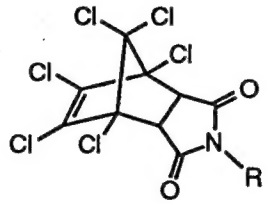
Activity assays. Access to large libraries of receptor molecules containing potential catalysts has made the development of efficient assays for catalytic activity of considerable importance. Clearly, the chances of finding a rare, but highly active, catalysts will increase with the number

of candidates that are screened. We have established the feasibility of using immunoassays (e.g., catELISA[7]) for screening bimolecular synthetic reactions. We demonstrated the utility of the catELISA with a bimolecular Diels-Alder reaction between an immmobilized dienophile and a diene in solution and explored the criteria for its successful application to other reactions.[8] This strategy promises to be a versatile and general solution to the screening problem.

RNA receptors. It has recently become possible to isolate RNA molecules that bind a variety of ligands from large pools of random sequences through an iterative selection and amplification process called SELEX.[9] To determine whether RNA molecules might have the same catalytic capabilities as antibodies, we selected a bimolecular Diels-Alder reaction for investigation. We previously showed that antibodies raised against the transition state analog **10** catalyze the cycloaddition between tetrachlorothiophene dioxide and *N*-alkylmaleimides.[10] This reaction is a good candidate for studies with RNA because a significant catalytic advantage might be achieved simply by holding the diene and dienophile close together. In collaboration with Larry Gold and his colleagues at the University of Colorado (Boulder) we have isolated RNA molecules that bind **10** ($K_d = 0.35 \pm 0.05$ mM) from a starting pool of $\approx 10^{14}$ sequences by affinity chromatography.[11] However, no catalysts were found among the binders. These results contrast with those with antibodies,[10] possibly because of the relatively low affinity of RNA for **10**. Although the Schultz group recently used the transition state analog approach to prepare a modestly active RNA catalyst for the racemization of a biphenyl derivative (another shape-selective reaction),[12] other strategies, such as selection on the basis of catalytic activity,[13] will likely be needed to identify oligonucleotides with the catalytic versatility of antibodies.



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SIGNIFICANCE: Enzymes use metal ions, vitamins, and cofactors to catalyze a wider spectrum of reactions than can be achieved using the 20 natural amino acids alone. Low molecular weight coenzymes are especially important in enzymic redox reactions and functional group transformations. Prosthetic groups also have the potential to expand the repertoire of reaction catalyzed by antibodies and other macromolecular catalysts. Moroever, because we are not restricted to biologically relevant cofactors, the properties of these systems can be optimized for high stability, catalytic efficiency, and low cost. Preparation of a peroxidase catalyst that depends for its activity on a benzene seleninic acid cofactor establishes the feasibility of this approach, and our preliminary results with the Hantzsch esters are very promising. However, several failures underscore how much we still have to learn about hapten design and how we are limited by our ability to screen the immune response effectively. The catELISA, invented by Green and coworkers for hydrolytic reactions and extended by ourselves to bimolecular synthetic reactions, provides a very powerful tool for addressing the latter problem, enabling a wide range chemical transformations to be assayed directly for catalysis.

PUBLICATIONS:

D. Hilvert (1993). Antibody catalysis of carbon-carbon bond formation and cleavage. *Acc. Chem. Res. 26*, 552-558.

G. MacBeath & D. Hilvert (1994). Monitoring catalytic activity by immunoassay: Implications for screening. *J. Am. Chem. Soc 116*, 6101-6106.

K.N. Morris, T.M. Tarasow, C.M. Julin, S. Simons, D. Hilvert & L. Gold (1994). Enrichment for RNA molecules that bind a Diels-Alder transition state analog. *Proc. Natl. Acad. Sci. USA*, in press.

PARTICIPATING SCIENTIFIC PERSONNEL

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Gavin MacBeath	10%
Theodore Tarasow	100%
Dr. Axel Neidlein	30%
Dr. Simon Thorn	30%
Dr. Magdalena Cid-Fernandez	100%
Dr. Jumi Shin	50%

Theodore Tarasow earned his Ph.D. from the Scripps Research Institute in May 1994 while employed on the project.

INVENTIONS:

None

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APPENDIXES

A. Reprints of: D. Hilvert (1993). Antibody catalysis of carbon-carbon bond formation and cleavage. *Acc. Chem. Res. 26*, 552-558.

B. Reprints of: G. MacBeath & D. Hilvert (1994). Monitoring catalytic activity by immunoassay: Implications for screening. *J. Am. Chem. Soc 116*, 6101.

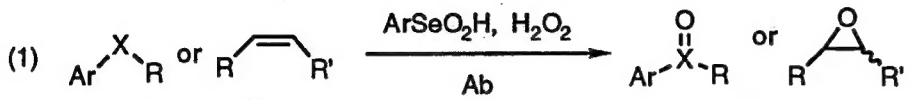
C. 1 Copy of the manuscript: K.N. Morris, T.M. Tarasow, C.M. Julin, S. Simons, D. Hilvert & L. Gold (1994). Enrichment for RNA molecules that bind a Diels-Alder transition state analog. *Proc. Natl. Acad. Sci. USA*, in press.

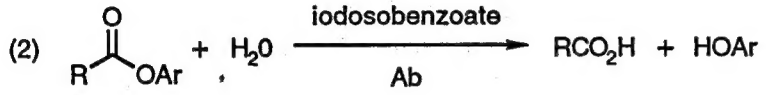
D. 1 Copy of: T.M. Tarasow, Tailored Biocatalysts for Carbon-Carbon Bond Forming and Cleaving Reactions. Ph.D. Thesis, 1994.

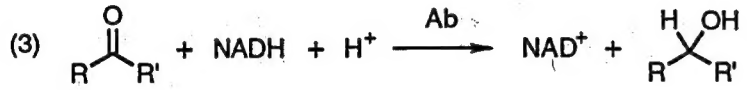
COFACTOR-ASSISTED ANTIBODY CATALYSIS

D. Hilvert, The Scripps Research Institute; 1994

Useful Cofactor-Dependent Reactions







Objectives

- Preparation of arylseleninic acid-dependent redox enzymes for efficient oxidation of sulfides, amines and olefins
- Preparation of *o*-iodosobenzoate-dependent antibodies for the hydrolysis of esters, amides and phosphate esters
- Preparation of antibody catalysts that utilize nicotinamide analogs for selective carbonyl reduction

Accomplishments

- Arylseleninic acid-dependent antibody catalyst identified and characterized
- Developed synthetic routes to nicotinamide transition state analogs based on Hantzsch esters
- Prepared antibodies that recognize a M^{II}(cyclen) complex of a phosphonotyrosine-containing peptide
- Developed an immunoassay (catELISA) for directly monitoring catalysis of a bimolecular reaction
- Evaluated RNA as an alternative to antibodies as a scaffold for creating tailored catalysts

Significance

- Cofactors promise to expand the repertoire of reactions catalyzed by antibodies
- In contrast to natural enzymes, non-biological cofactors that are inexpensive, easily prepared and highly stable can be exploited
- Establish structure-function relationships for protein catalysis

Reprinted from Accounts of Chemical Research, 1993, 26.

Antibody Catalysis of Carbon–Carbon Bond Formation and Cleavage

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Received May 3, 1993

Nearly a century ago, the lock-and-key description of enzyme action was formulated by Emil Fischer.[1] When modified to account for the possibility of ligand and protein conformational changes, this simple metaphor still conveys the basic principle of catalysis: that each enzyme must possess an "active site" tailored for recognition and stabilization of the rate-limiting transition state of the reaction it promotes. This metaphor can also be applied to the field of catalytic antibodies,[2] in which rationally designed haptens unlock the catalytic potential of the humoral immune system.

Preparation of catalytic antibodies exploits the immune system's sophisticated ability to produce selective binding pockets for almost any molecule of interest. To induce a binding site with a topology and stereoelectronic environment suitable for catalysis, a stable molecule which mimics the structure of the short-lived transition state of the target reaction is employed as a hapten. This approach to tailored catalyst molecules is very general, since transition-state analogs for many reaction types can be designed using an understanding of chemical mechanism and the basic principles of physical organic chemistry. In the last six years, well over 50 reactions have been accelerated by antibodies.[2] These include simple hydrolytic reactions, pericyclic processes lacking biological counterparts, and transformations that are otherwise chemically disfavored. In addition to transition-state analog design, two less widely-appreciated factors are also critical to the success of a catalytic antibody experiment: the use of monoclonal antibodies and the application of effective screening methods. Given the many ways in which an asymmetric molecule can be bound by a protein, only a small fraction of the many different antibodies that recognize an individual hapten may possess the desired catalytic properties. Rapid assay methods substantially increase the probability of finding an efficient catalyst by facilitating the preliminary evaluation of large numbers of possible candidates, while monoclonal antibody technology permits the reproducible study of individual immunoglobulins in pure form.

The field of catalytic antibodies has been extensively reviewed in recent years.[2] Rather than provide another broad overview of this subject, the present Account will focus on a few transformations involving the formation and cleavage of carbon–carbon bonds. Although the availability of a large number of well-characterized transition-state analogs for hydrolytic reactions has made them the primary targets of antibody catalysis to date, reactions in which carbon atoms are connected or disconnected may be of greater intrinsic interest to the synthetic organic chemist. These transformations are crucial to the survival of all living organisms. They are also central to *in vitro* synthetic strategies leading to the construction of useful molecules of all kinds, from relatively simple commodity chemicals to novel polymeric materials and densely-functionalized natural products. Because the regio- and stereoselectivity of C–C bond forming reactions is often difficult to control using conventional methodologies, the creation of tailored antibody catalysts may yield useful new tools for asymmetric synthesis. For example, the aldol reaction remains one of the most powerful methods for forming C–C bonds; antibodies with significant aldolase activity would greatly extend current chemoenzymatic approaches[4] to the synthesis of sugars and other compounds via this reaction.

Aside from their practical value, reactions in which C–C bonds are made or broken are also mechanistically interesting and can be used to illuminate some of the basic strategies which enzymes exploit to reduce free energies of activation. Enzymes are thought to achieve their remarkable catalytic effects through a combination of substrate destabilization, proximity effects, and chemical catalysis mediated by appropriately positioned nucleophiles, general acids and bases, metal ions, and other diverse cofactors.[5] However, the contribution of individual factors to the overall efficiency of an enzyme-catalyzed reaction has been difficult to measure experimentally because of the inherent complexity of these systems. Catalytic antibody technology now makes it possible to examine specific mechanistic issues in the absence of competing effects by using tailor-made active sites and model reactions that are particularly sensitive to one factor or another. The knowledge gained in the course of these studies has important implications for the (re)design of transition-state analogs. This knowledge is also essential for the development of general

Donald Hilvert is a member of the Departments of Chemistry and Molecular Biology at The Scripps Research Institute. He received his B.A. and Ph.D. (1983) from Brown and Columbia Universities, respectively, and was a postdoctoral fellow at Rockefeller University. His research interests include the design of semisynthetic enzymes, catalytic antibodies, and molecular evolution. Dr. Hilvert was a recent recipient of an Arthur C. Cope Scholar Award from the American Chemical Society.

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rules that will guide and improve future efforts to capitalize on the immune system's "hidden" capacity for catalysis.

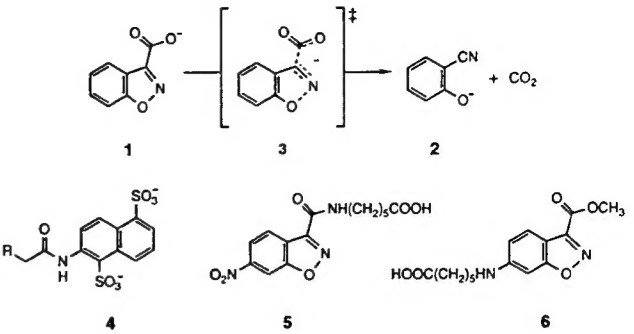
Decarboxylations and Substrate Destabilization

Carboxylation and decarboxylation reactions are crucial components of aerobic and anaerobic metabolism, as exemplified by the fixation of carbon dioxide in photosynthesis and the decarboxylation of α- and β-ketoacids in the Krebs cycle. Most enzymes that promote these reactions require metal ions or a cofactor such as thiamine pyrophosphate to effect catalysis.[6] However, substrate destabilization (through desolvation of the negatively charged carboxylate moiety bound at the active site) is also thought to contribute to the overall catalytic efficiency of these agents.[6,7] Lienhard showed some years ago that the thiamine-catalyzed decarboxylation of α-ketoacids could be greatly enhanced by transferring the reaction from water to nonaqueous solvents.[7] More recently, crystallographic studies of histidine decarboxylase from *Lactobacillus 30a* have established that the carboxylate binding site is predominantly hydrophobic.[8]

To probe the importance of medium effects and desolvation in catalysis, we have examined a mechanistically simple decarboxylation reaction.[9] The unimolecular conversion of 3-carboxybenzisoxazoles (**1**) into salicylonitriles (**2**) is a concerted, intermediateless process that proceeds through a charge-delocalized transition state (**3**)[10] (see Scheme I). This reaction is remarkably sensitive to solvent microenvironment; its reaction rate increases as much as 10^8-fold upon transfer of the reactant from aqueous solution to aprotic dipolar solvents. This dramatic range of reactivity has been attributed mainly to two factors: destabilization of the ground-state carboxylate upon removal of hydrogen-bonding interactions with water, and concomitant stabilization of the charge-delocalized transition state in organic solvents through dispersion interactions.[10] The rate-retarding effect of ion-pair formation in solvents of low dielectric constant has also been noted.[10,11]

We employed the napthalene disulfonate derivative **4** to generate a number of monoclonal antibodies capable of catalyzing the decarboxylation of 5-nitro-3-carboxybenzisoxazole.[9] The best of these, 21D8, accelerates this reaction 19 000-fold over the rate in aqueous buffer. The successful hapten (**4**) combined a large aromatic moiety and anionic substituents in order to elicit an appropriately-sized apolar pocket containing complementary positively-charged residues that might facilitate anion binding. In related experiments with haptens **5** and **6**, which contain a neutral amide and ester in place of the carboxylate group, no catalysts

Scheme I



were obtained.[9] In the latter cases, it is possible that the available binding energy was insufficient to bury the substrate carboxylate in a hydrophobic pocket.

Like a typical enzyme, 21D8 displays saturation kinetics and multiple turnovers. The cleavage of the carbon–carbon bond is fully rate-determining, as judged by measuring the carbon kinetic isotope effect (k^{12}/k^{13}) on the rate of the antibody-catalyzed decarboxylation.[12] Moreover, similar carbon kinetic isotope effects for the reaction in water, in dioxane, and in the active site of 21D8 indicate that transition-state structure does not change significantly when the solvent microenvironment is altered,[12] even though the rate of reaction varies by a factor of $>10^4$.

Decarboxylation of 3-carboxybenzisoxazoles is not subject to general acid–base catalysis or to stereochemical constraints.[10] Hence, the large rate acceleration provided by the antibody can be ascribed almost entirely to medium effects. This conclusion is supported by experiments using fluorescent hapten derivatives as reporter groups. These studies showed that the antibody binding pocket is apolar and virtually inaccessible to water when occupied by a ligand.[9] Analysis of the thermodynamic activation parameters also indicates that antibody catalysis of the decarboxylation, like the solvent-associated rate acceleration, is due to a more favorable enthalpy of activation ($\Delta\Delta H^{\ddagger} = -8.1$ kcal/mol). Unlike reactions in organic solvents, the antibody's entropy of activation is significantly less favorable than that of the spontaneous reaction in water ($-T\Delta\Delta S^{\ddagger} = +2.7$ kcal/mol).[9] These findings suggest that the antibody provides a rigid microenvironment for the reaction. Unlike free solvent, this environment cannot relax as the charge-delocalized transition state is approached.

Because the antibody's discrete and chemically well-defined structure is amenable to genetic alteration through site-directed mutagenesis, the specific factors influencing its ability to desolvate the substrate and accelerate the chemical reaction (e.g., dipolarity, electrostatics, and hydrogen bonding) can now be evaluated in a systematic fashion. It may also be possible to increase the potency of the catalyst through protein engineering. Multiparametric analysis of the rates at which 3-carboxybenzisoxazoles decarboxylate in 20 solvents having widely different properties indicates that solvent hydrogen bond acidity is likely to be the dominant rate-retarding factor.[13] If the rate enhance-

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ment achieved by 21D8 is limited by interactions between the carboxylate and a hydrogen bond donor at the active site, repositioning or removing this residue to prevent the deleterious interactions might augment activity without compromising substrate binding.

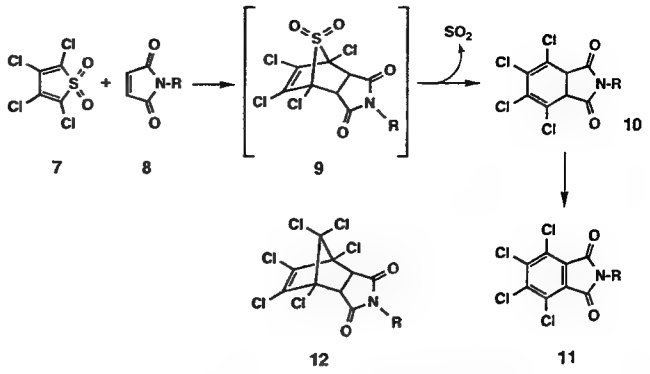
Many important chemical transformations, including aldol condensations, S_N2 substitutions, E2 eliminations, and even some hydrolytic reactions, can be greatly accelerated by altering their solvent microenvironment. They should therefore be particularly susceptible to antibody catalysis. Furthermore, desolvation of charged groups represents only one of several mechanisms by which substrate destabilization can be achieved. Geometric strain or distortion and electrostatic repulsion are also effective, potentially yielding very large rate accelerations, provided that the induced destabilization can be fully relieved at the transition state.[5] Cochran and Schultz have already exploited geometric strain to generate mimics of the enzyme ferrochelatase:[14] antibodies raised against an *N*-methylated porphyrin, which has a nonplanar structure corresponding to a distorted substrate conformation, substantially accelerated the metallation of mesoporphyrins by Zn^{2+}. Amide hydrolysis might be enhanced in a similar fashion if binding energy can be harnessed so as to force the amino group out of conjugation with the scissile carbonyl.[15] The catalytic advantage to be derived from substrate destabilization is limited only by the amount of binding energy available to force the substrate molecule into the destabilizing environment. Thus, this strategy is likely to be increasingly exploited in the development of a wide variety of catalytic antibodies.

Diels–Alder Reactions and Catalysis by Approximation

Many chemical reactions are limited by high entropic barriers. For example, bimolecular versions of the synthetically useful Diels–Alder reaction, in which an olefin adds to a conjugated diene to give a cyclohexene product, typically have an unfavorable entropy of activation ($\Delta S^{\ddagger}$) in the range −30 to −40 cal K^{-1} mol^{-1}. One of the simplest ways in which an enzyme can speed such a process is to act as an "entropy trap", utilizing binding energy to bring the two substrate molecules together in the correct orientation for reaction.[5] This is sometimes referred to as catalysis by approximation.

Despite the importance of the Diels–Alder reaction to organic synthesis,[16] enzymes that catalyze such cycloadditions have not yet been found in Nature. For this reason, recent demonstrations[17,18] that antibodies can promote these reactions are an exciting advance. Here, suitable hapten design is dictated by the boatlike structure of the uncharged, highly-ordered Diels–Alder transition state. This species resembles the cyclohexene product more than it does the individual starting materials. However, the product itself is generally a poor choice of hapten,[19] given the likelihood of severe

Scheme II



product inhibition in the induced binding pockets, and novel strategies must be developed to facilitate catalyst turnover.

One way to minimize product inhibition draws on chemical or conformational change to drive product release. We used this approach to catalyze the Diels–Alder reaction between tetrachlorothiophene dioxide (**7**) and *N*-alkylmaleimides (**8**)[17] (see Scheme II), a reaction that occurs in two steps, with the initially formed tricyclic adduct **9** undergoing facile chelotropic elimination of sulfur dioxide to give the dihydrophthalimide **10**. The latter is subsequently oxidized to **11** under the reaction conditions. The transition states for both the cycloaddition and the cycloreversion resemble the high-energy intermediate **9**. The stable hexachloronorbornene derivative **12** mimics key geometric features of this species, including the boat conformation of the cyclohexene ring. It was used to generate antibodies that catalyze the reaction between **7** and **8** efficiently, with substantial rate accelerations and multiple turnovers.

The effective molarity for one of these antibodies, 1E9, was determined to be in excess of 10^2 M from comparison of the pseudo-first-order rate constant for the reaction at the antibody active site (k_{cat}) with the second-order rate constant for the uncatalyzed process in free solution (k_{un}).[17] This value indicates the concentration of substrate that would be needed in the uncatalyzed reaction to achieve the same rate as seen in the enzyme ternary complex and thus provides a measure of catalytic efficiency. Page and Jencks have estimated that the maximum kinetic advantage to be derived from converting a bimolecular to a pseudo-unimolecular reaction is on the order of 10^8 M for 1 M

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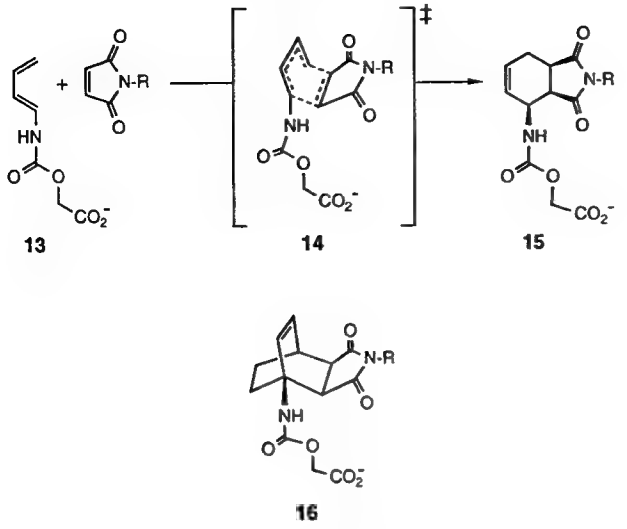
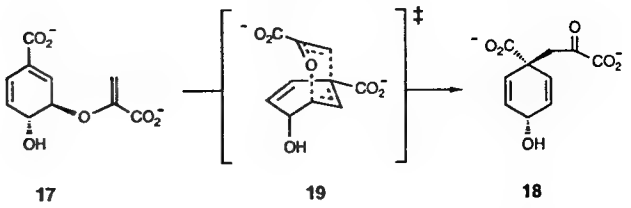
(18) Braisted, A. C.; Schultz, P. G. *J. Am. Chem. Soc.* **1990**, *112*, 7430.

(19) Suckling and co-workers recently reported using a product analog for a Diels–Alder reaction to raise antibodies capable of accelerating the reaction between acetoxybutadiene and an *N*-alkylmaleimide (Suckling, C. J.; Tedford, M. C.; Bence, L. M.; Irvine, J. I.; Stimson, W. H. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 49). However, several observations raise doubt about the claims made for this process. First, although Diels–Alder reactions between neutral substrates are expected to be independent of pH, the rate of cyclohexene formation in the presence of their antibody increased dramatically as the pH was raised to 9. Second, the putative antibody-catalyzed cycloaddition was not inhibited by relatively high concentrations of product (0.45 mM), suggesting that the reaction did not occur in the induced antibody combining site. Rapid hydrolysis of the acetoxybutadiene, brought about by the contaminating esterase that was detected in the antibody samples, would yield a much more reactive diene for the spontaneous reaction with maleimide. This explains the available data, including the isolation of a cyclohexenol (rather than the corresponding acetyl ester) as the major product of the reaction. This alternative mechanism also accounts for the surprising finding that nonhydrolyzable methoxybutadiene is not a substrate for the antibody, even though it is less sterically demanding and intrinsically more reactive than acetoxybutadiene.

Scheme III



Scheme IV



standard states.[20] While 1E9 does not approach this level of efficiency, its effective molarity is still 5 orders of magnitude larger than the solubility limit of tetrachlorothiophene dioxide in the reaction medium.

Structural work on 1E9 is at an early stage, but sequencing experiments[21] have shown that this immunoglobulin has high homology with the progesterone-binding antibody DB3.[22] Crystallographic studies of DB3 have revealed a very hydrophobic binding pocket that almost completely envelops its steroid ligand.[22] A similar active site geometry can be inferred for 1E9. Kinetic experiments have shown that binding interactions to nonreacting, hydrophobic portions of the maleimide substrate substantially increase catalytic efficiency. Thus, *N*-alkylmaleimides with long alkyl side chains are better substrates than maleimides with short side chains (i.e., R = butyl ≈ propyl > ethyl > methyl ≫ H).[23] Kinetic studies[23] have also established that the spontaneous elimination of SO_2 from **9** is rapid and that the final product of the reaction is bound only weakly by the antibody, presumably because of poor shape complementarity. The dissociation constant for *N*-ethylphthalimide was determined to be 0.5 mM, only 40 times smaller than that of *N*-ethylmaleimide (K_m = 18 mM). The hapten (K_i = 8 nM), on the other hand, binds nearly 5 orders of magnitude more avidly than the phthalimide and 6 orders of magnitude more tightly than either reactant. Together, these results provide strong validation for the principles used to design the hapten for these experiments.

Braisted and Schultz adopted a similar strategy to catalyze the Diels–Alder reaction between the acyclic diene **13** and *N*-phenylmaleimide[18] (see Scheme III). They synthesized the bicyclooctene hapten **16** as a mimic of transition-state structure **14**. The analog contains an ethano bridge (rather than the CCl_2 unit in **12**) to lock the cyclohexene ring into a boat conformation. In this case, the product's tendency to undergo an energetically favorable conformational change, rather than a second chemical reaction, was expected to reduce its affinity for the active site. Antibody 39,-A11 was raised against **16** and accelerates the target reaction with multiple turnovers. As expected, product binds only 75–100 times more tightly than either substrate, but the catalyst is rather inefficient as judged by its low effective molarity (0.35 M). These results indicate poor use of antibody binding energy. It is possible that the active site generated by the [2,2,2]-bicyclooctene is simply too large to completely freeze out the degrees of freedom available to a substrate diene lacking a structural equivalent of the ethano bridge. More extensive screening of the immunological response to haptens like **16** will probably be necessary to find catalysts with high activity.

Virtually all bimolecular reactions will be amenable to antibody catalysis through proximity effects. Moreover, the use of antigens resembling high-energy product conformers or unstable reaction intermediates is likely to be a general solution to the problem of product inhibition in these reactions. Extension of these ideas to new systems consequently has the potential to provide versatile catalysts for a variety of practical applications. Given the precise control over ligand binding that antibodies exercise, for example, antibodies reversing normally observed Diels–Alder endo/exo ratios or favoring rare product regioisomers should be accessible.

Sigmatropic Rearrangements and in Vivo Applications

Recent progress in producing immunoglobulins in microorganisms such as *Escherichia coli* and yeast opens the door to many exciting applications of catalytic antibodies *in vivo*. For instance, it may be possible to engineer new properties such as pesticide resistance or unusual biosynthetic capabilities into living organisms by intracellular synthesis of these agents. Alternatively, given their biocompatibility and selectivity, tailored antibody catalysts might some day be used to complement human metabolic disorders.

The rearrangement of (–)-chorismate **17** into prephenate **18** (see Scheme IV), which is a key step in the biosynthesis of the aromatic amino acids tyrosine and phenylalanine in plants and lower organisms (Figure 1),[24] is an excellent model system for evaluating the feasibility of antibody catalysis *in vivo*. This reaction, formally a Claisen rearrangement, is a rare example of a pericyclic process in primary metabolism. The enzyme chorismate mutase accelerates the reaction by more than 10^6-fold and is essential for normal cell growth on minimal medium. Strains of yeast or *E. coli* engineered to lack an active version of this enzyme are auxotrophic for tyrosine and phenylalanine.

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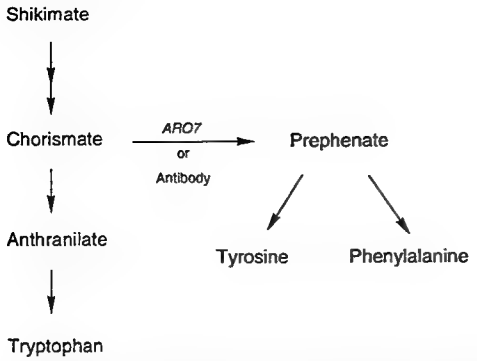
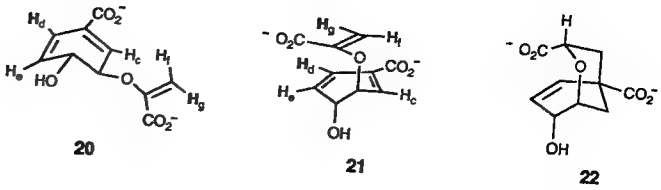


Figure 1. Shikimate pathway for the biosynthesis of aromatic amino acids in yeast. *ARO7* encodes the enzyme chorismate mutase, which promotes the rearrangement of chorismate into prephenate.

While the uncatalyzed rearrangement of chorismate has been well studied, the detailed mechanism of the mutase reaction is still unknown. Like the Diels–Alder reaction discussed in the previous section, this sigmatropic rearrangement should be sensitive to proximity effects. Stereochemical studies have established that both the enzyme-catalyzed and spontaneous reactions occur via a compact, chairlike transition state (**19**).[25] In aqueous solution, however, the substrate adopts an extended conformation (e.g., **20**) and must undergo a conformational change to position the enolpyruvate side chain properly for reaction.[26] Given this, it has been proposed[27,28] that the enzyme may increase the probability of reaction by selectively binding and stabilizing the reactive pseudo-diaxial conformer **21**. The very favorable entropy of activation for the enzyme-catalyzed rearrangement ($\Delta S^{\ddagger} \approx 0$ cal K^{-1} mol^{-1}) compared to that of the spontaneous reaction ($\Delta S^{\ddagger} = -12.9$ cal K^{-1} mol^{-1}) is consistent with this suggestion.[28] Considerable dipolar character in the transition state has also been inferred from isotope effect studies,[29] with C–O bond cleavage preceding C–C bond formation. Thus, electrostatic and/or hydrogen-bonding interactions may also contribute to the enzyme's overall catalytic efficiency.[30]

The oxabicyclic dicarboxylic acid **22** has been used in two different laboratories to raise antibodies with chorismate mutase activity.[31,32] This compound resembles the conformationally restricted transition-state structure **19** and is currently the most potent inhibitor of chorismate mutase available.[33] Antibody 11F1-2E9 was elicited with this compound by Schultz and coworkers[31] and shown to effect a remarkable 10^4-fold acceleration of the rearrangement (k_{cat}/k_{un}). The catalytic efficiency is only 10^2 times smaller than that



20 **21** **22**

achieved by the natural *E. coli* enzyme under identical conditions. As seen with chorismate mutase itself, the antibody-catalyzed reaction is characterized by a favorable $\Delta S^{\ddagger}$ (–1.2 cal K^{-1} mol^{-1}) relative to the uncatalyzed reaction,[34] illustrating the importance of entropic effects.

A second chorismate mutase antibody (1F7), developed in our laboratory,[32] is roughly 10^2 times less active than 11F1-2E11. Desite its relatively sluggish activity, 1F7 is highly enantioselective, showing a >90:1 preference for rearrangement of the natural (–)-isomer of chorismate, a property that was successfully exploited in the kinetic resolution of racemic substrate.[35] In contrast to 11F1-2E9, 1F7 achieves its catalytic effects entirely by lowering the enthalpy of activation.[32] The entropy of activation is actually 10 cal K^{-1} mol^{-1} *less* favorable than for the spontaneous thermal rearrangement. While detailed interpretations of activation parameters obtained for reactions carried out in organized media are notably unreliable, the latter observation raises interesting questions regarding the conformation chorismate adopts at the active site of 1F7. To address this issue, we have carried out an NMR study using transferred nuclear Overhauser effects (TRNOE's).[36] Clear evidence was obtained for magnetization transfer between H_g and H_d and between H_f and H_c when chorismate was bound at the antibody active site. These results are diagnostic for the pseudodiaxial conformer **21** and indicate that the antibody pocket is able to preorganize its substrate to an appreciable extent, as dictated by the structure of the templating hapten. The detection of smaller, nonspecific TRNOE's in these experiments suggests, however, that 1F7 may not completely reduce the rotational degrees of freedom available to the enolpyruvyl group. The ability of the substrate to bind in catalytically-unproductive modes may account in part for the antibody's relatively low efficiency compared to 11F1-2E9 and natural chorismate mutase.

The isolation of two different antibodies with chorismate mutase activity underscores the importance of screening the immune response to a given hapten as widely as possible. Given the enormous diversity of the immunological repertoire, the probability of finding an effective catalyst will generally rise with the number of antibodies screened. In the chorismate mutase experiments, only about 50 hapten binders were examined, representing a tiny fraction of the estimated 10^7–10^8 antibody receptors potentially available for ligand recognition prior to somatic diversification.[3] By assaying larger numbers of hapten binders, catalysts even more active than 11F-2E11 might conceivably be found. It might also be possible to find antibodies that

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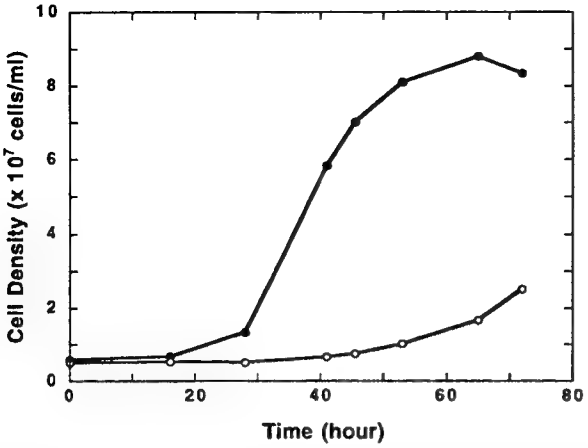

Figure 2. Growth curves[40] for a chorismate mutase-deficient yeast strain (351m), harboring (●) or lacking (○) a plasmid encoding the Fab fragment of the chorismate mutase antibody 1F7. The cells were grown under selective conditions at 30 °C in liquid medium lacking phenylalanine.

promote the stereospecific rearrangement of (+)-chorismate, since the transition-state analog 22 used in these experiments was racemic.

The distinctive kinetic and thermodynamic properties of the two chorismate mutase antibodies discussed here also illustrate the ability of the immune system to solve a specific catalytic task in a variety of interesting ways. This mechanistic versatility has also been noted in the context of hydrolytic antibodies, some of which exploit nucleophilic catalysis while others do not.[37] The precise structural basis for the differences between 1F7 and 11F1-2E9, and the features that differentiate them from the natural enzyme, will eventually be resolved through X-ray crystallographic studies. In this regard, recent successes growing small crystals of the Fab′ fragment of 1F7 that diffract to about 3.0-Å resolution represent a promising development.[38] The structure of the antibody–hapten complex is currently being solved by molecular replacement methods and will undoubtedly yield valuable insights into structure–function relationships in this catalyst.

In complementary molecular biological studies, the genes encoding the heavy and light chains of 1F7 have been cloned, sequenced, and engineered for intracellular expression in *Saccharomyces cerevisiae* as an Fab fragment.[39] Under optimal conditions, the antibody can be produced at levels corresponding to ca. 0.1% of total cellular protein in a variety of yeast strains lacking natural chorismate mutase. The yeast-derived Fab not only folds and assembles correctly within the cell, but its binding and catalytic properties *in vitro* are indistinguishable from those of the parent immunoglobulin.[39] More importantly, the Fab fragment has been shown to confer a substantial growth advantage to a permissive chorismate mutase-deficient host strain under auxotrophic conditions (Figure 2).[40] In control experiments, an antibody with esterolytic activity was unable to complement the host's chorismate mutase deficiency. These experiments demonstrate that antibodies can function *in vivo* to catalyze an essential metabolic transformation and thereby reconstitute a defective biosynthetic pathway. The ability to select for a phenotype altered in a predictable fashion also provides the means for increasing the activity of the first-generation catalyst. 1F7 is 4 orders of magnitude less potent than natural chorismate mutase. Through extensive random mutagenesis of the antibody-encoding genes and genetic selection it should be possible to exploit the host cell's requirement for antibody activity to identify 1F7 variants with increased potency. Experiments along these lines are currently underway in our laboratory.

Next Steps

As the above examples illustrate, catalytic antibody technology provides the means to create a wide variety of enzyme-like catalysts possessing tailored activities and specificities. Even reactions lacking physiological counterparts can be catalyzed. If the full promise of this technology is to be realized, however, a number of practical problems will have to be solved. The principal challenge for the future will be the production of truly efficient catalysts. Although large rate accelerations have been observed in some cases, most catalytic antibodies exhibit relatively low activities. Even the best cases fall several orders of magnitude short of the rate enhancements achieved by analogous naturally-occurring enzymes.

This low efficiency is not surprising given that stable haptens can never mimic transition structures perfectly. Antibodies raised against imperfect transition-state analogs cannot be expected to reduce free energies of activation to the same extent that authentic enzymes do. The challenge of effective hapten design is made even greater by the fact that many energetically demanding reactions require induction of multiple catalytic groups. Simple strategies exploiting charge complementarity to elicit general acids, general bases, and nucleophiles at the antibody combining site (the "bait-and-switch" approach) have been developed to address this problem,[41] but arrays of residues as sophisticated as the catalytic triad in serine proteases may be difficult to generate with a single hapten. In addition, for most reactions, transition-state analog design has not been optimized. Even for those reactions for which excellent haptens are available, such as ester hydrolysis, a correlation between hapten structure and the probability of inducing an active antibody has not been established in a statistically rigorous fashion. Complete, careful characterization of existing catalytic antibody active sites may help identify the important structural features of individual haptens that can improve the odds of generating highly active catalysts. Incorporation of metal ions[42] and other catalytic

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cofactors[43] into the antibody pocket may also extend the properties of these agents.

Another means of obtaining more efficient antibodies requires providing better access to the full diversity inherent in the immune repertoire. Over the last six years, the majority of the catalytic antibodies that have been reported were prepared using standard hybridoma techniques.[44] Given the difficulty and expense of maintaining large numbers of hybridomas, only a fraction of the immune response to any hapten has been assayed for catalysis. As the most active catalysts may also be the rarest, far larger numbers of antibodies will have to be screened to ensure that the best molecules are not missed. In fact, large combinatorial libraries of antibody fragments can now be generated readily and rapidly using the polymerase chain reaction (PCR).[45] When displayed on the surface of the filamentous phage virion, the desired active sites can be selected on the basis of antigen affinity.[46] If coupled with sensitive colorimetric or biological assays, this new technology is likely to be an enormously valuable tool in the search for highly active antibodies. By increasing the number of hapten binders screened for catalytic activity, it will also be possible to assess the efficacy of individual hapten molecules in a statistically rigorous way.

Finally, since catalytic antibodies with modest activity are now readily available, general strategies for augmenting their chemical efficiency directly must be developed. For that task, modern genetic techniques hold the greatest promise. Natural enzymes have been brought to peak efficiency through millions of years of evolution. It may be possible to adopt an evolutionary approach in the laboratory by subjecting first-generation antibody catalysts to extensive random mutagenesis and identifying improved variants by classical genetic selection. Strong selection assays can be devised for a broad range of transformations, including any metabolic reaction (e.g., reactions leading to the synthesis of an essential nutrient, drug, or hormone) and reactions that destroy pesticides or other toxins. As already mentioned, the viability of this approach is currently being tested with the chorismate mutase antibody 1F7. Characterization of the genetic changes that accumulate as the antibody's activity increases will provide fundamental insights into the evolution of molecular function and the relationship between structure and activity. Site-directed mutagenesis, on the other hand, is likely to become an increasingly important tool for probing and improving the properties of these molecules as detailed structural information becomes available for individual antibody combining sites.

Progress in the field of catalytic antibodies has been rapid over the past six years and is likely to continue apace. By learning more about fundamental structure–function relationships in these molecules and by optimizing our strategies for preparing them, we expect to be able to create immunoglobulins that rival the efficiency of naturally-occurring enzymes.

The author gratefully acknowledges the NIH (GM38273), NSF (DMB-8912068), ARO, ONR, and the American Cancer Society (FRA-60465) for financial support. He is a fellow of the Alfred P. Sloan Foundation.

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Reprinted from the Journal of the American Chemical Society, 1994, *116*.

Monitoring Catalytic Activity by Immunoassay: Implications for Screening

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Received December 20, 1993®

Abstract: We have developed an immunoassay for screening large libraries of potential catalysts of a bimolecular Diels–Alder reaction. The procedure involves the reaction of immobilized dienophile with diene in solution and subsequent detection of the product with a monoclonal antibody as in a regular enzyme-linked immunosorbant assay (ELISA). The assay exhibits the expected dependence on time and diene concentration. A catalytic antibody accelerates the reaction in a concentration dependent manner despite immobilization of the dienophile, and its catalytic efficiency is comparable to that in free solution. The current study explores both the utility of this assay and criteria for its successful application to other reactions. Employment of this strategy, previously dubbed catELISA (Tawfik, *et al. PNAS* **1993**, *90*, 373), to monitor a bimolecular cycloaddition reaction supports its potential as a general approach to screening for catalysts.

Rational approaches to catalyst design, including catalytic antibodies, are being pursued in a number of research groups.[1] Many of these strategies entail the generation of large libraries of variants biased in some manner to contain catalysts (for example, by selecting molecules that bind transition-state analogs). To optimize the chances of identifying and isolating highly efficient catalysts, it is desirable to evaluate as many candidates from the biased libraries as possible. Consequently, the development of efficient and facile assays for screening large numbers of molecules for catalytic activity is of considerable importance.

The most common method of assaying catalytic activity relies on the release of a chromophore as the reaction proceeds. While this approach has been exploited successfully for screening in many instances,[2-4] the range of substrates that can be studied is relatively limited. Alternative, yet sensitive, means of product detection can obviate the need for chromophoric substrates. For example, Schwabacher and co-workers[5] have recently developed an assay for bimolecular reactions in which an easily detected tag, like biotin, becomes incorporated into the product during the reaction. A potentially more general approach has been reported by Green and co-workers[6] in which product is detected by specific antibodies as in a regular enzyme-linked immunosorbent assay (ELISA).[7,8] The authors used the assay, which they dubbed catELISA, to monitor the hydrolysis of an immobilized ester and identified nine catalytic antibodies from a total of 1570 hybridoma clones.

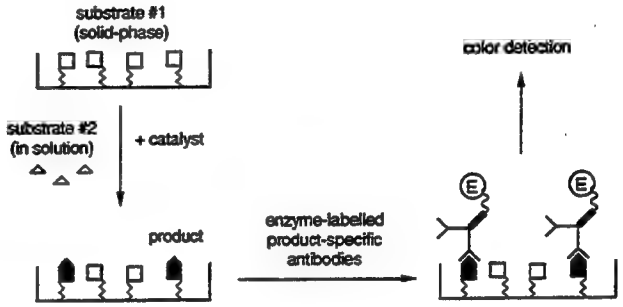

Figure 1. Strategy of the bimolecular catELISA. Substrate #1 is immobilized on a microtiter plate. Substrate #2 is then added in solution, along with the putative catalyst being screened. Catalytic activity results in formation of immobilized product, which is then detected using a product-specific antibody. An enzyme covalently linked to this antibody catalyzes a reaction with a chromophoric substrate.

The effectiveness of the catELISA approach has thus been demonstrated for a hydrolytic reaction in which strongly antigenic determinants on the substrate are removed during the reaction. In order to evaluate the generality of this assay, we sought to extend the strategy to synthetic, bimolecular reactions (Figure 1). For this study, we chose the Diels–Alder reaction of Figure 2, which represents a particularly promising aspect of rational catalyst design: the development of enzyme-like macromolecules that catalyze classes of reactions for which natural enzymes are unavailable. Application of the catELISA to this reaction enables us to assess the accuracy of the assay in estimating catalytic efficiency and to evaluate the factors required for successful implementation of this technique.

Materials and Methods

All chemicals, unless otherwise stated, were obtained from Aldrich and used without further purification. The catalytic antibody 1E9 was prepared and purified as previously described.[9] The substrate tetrachlorothiophene dioxide (TCTD) was prepared

* Abstract published in *Advance ACS Abstracts*, May 15, 1994.

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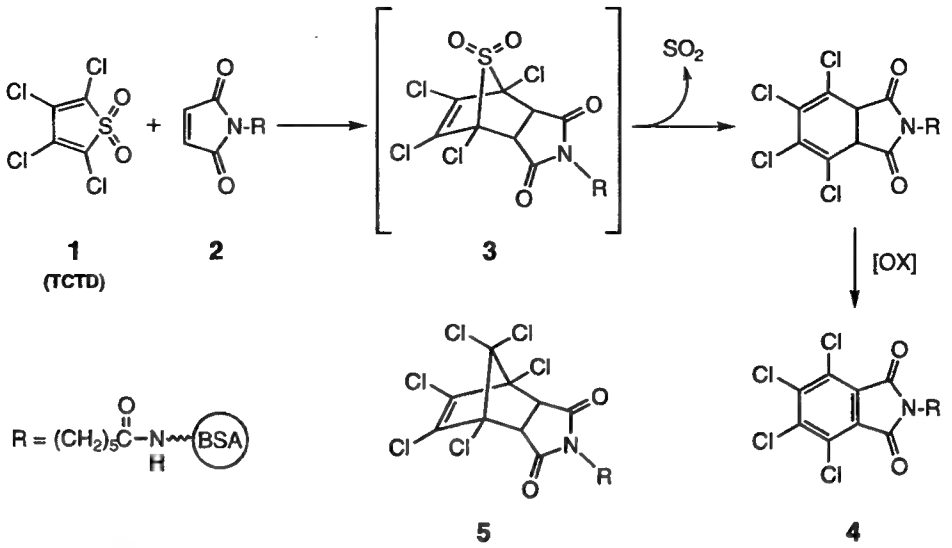

Figure 2. Diels–Alder reaction of tetrachlorothiophene dioxide (**1**) with *N*-derivatized maleimide (**2**). The transition state of the reaction (similar to **3**) is mimicked by the analog **5**.

according to the method of Raasch.[10] Flash chromatography was performed by the method of Still, Kahn, and Mitra[11] using 60-mesh silica gel. NMR spectra were obtained with Fourier transform spectrometers operating at the indicated frequencies and were referenced to the residual peak of the indicated solvent. Mass spectra were obtained on a VG ZAB-2VSE double-focusing high-resolution mass spectrometer.

Maleimide–Protein Conjugates. 6-Maleimidocaproic acid was synthesized as reported by Keller and Rudinger.[12] To a solution of 125 mg (0.59 mmol) of 6-maleimidocaproic acid and an excess of triethylamine in 10 mL of dichloromethane was added an excess of disuccinimidyl carbonate and the mixture stirred for 2 h under N_2 at room temperature. The succinimidyl ester was recovered by evaporation of solvent. Purification (silica gel, elution with 1:1 ethyl acetate/dichloromethane) gave 165 mg (90%). ^{1}H-NMR: ($CDCl_3$, 300 MHz) δ 6.67 (s, 2H), 3.50 (t, 2H, J = 7.0 Hz), 2.79 (s, 4H), 2.58 (t, 2H, J = 7.6 Hz), 1.79–1.69 (m, 2H), 1.65–1.55 (m, 2H), 1.44–1.34 (m, 2H). MS m/z (LSIMS$^+$): calcd 309.1087, obsd 309.1093.

The succinimidyl ester (20 mg, 65 μmol) was dissolved in 0.40 mL of 1,4-dioxane and added to 43 mg (0.65 μmol) of bovine serum albumin (BSA) in 10.6 mL of 100 mM $NaHCO_3$(aq), pH 8.0. After 2 h at room temperature, the BSA conjugate was purified on a Sephadex G-50 column, eluting with MBS-6.0 (20 mM MES (2-[*N*-morpholino]ethanesulfonic acid), 100 mM NaCl, pH 6.0). Amine titration[13] indicated 44 haptens/BSA molecule. The conjugate was stored at −70 °C.

Tetrachlorophthalimide–Protein Conjugates. To a solution of 1.0 g (3.5 mmol) of tetrachlorophthalic anhydride in 50 mL of ethyl acetate was added 500 mg (3.8 mmol) of 6-aminocaproic acid. After refluxing under argon for 1 h, 2.2 g (28 mmol) of acetyl chloride was added and the mixture refluxed for an additional hour. The mixture was filtered and the filtrate washed once with 1 N HCl(aq) and once with saturated NaCl(aq) and dried with $MgSO_4$. Evaporation of solvent gave 1.18 g (85%) of 95% pure 6-(*N*-tetrachlorophthalimide)caproic acid. Further purification was achieved by silica gel chromatography, eluting with ethyl acetate. ^{1}H-NMR: (DMSO-d_6, 300 MHz) δ 11.02 (s, 1H), 3.54 (t, 2H, J = 7.1 Hz), 2.18 (t, 2H, J = 7.3 Hz), 1.62–1.45 (m, 4H), 1.32–1.22 (m, 2H). ^{13}C-NMR: (DMSO-d_6, 500 MHz) δ 174.4, 163.5, 138.0, 128.4, 128.0, 38.1, 33.4, 27.3, 25.7, 24.0. MS m/z (LSIMS$^-$): calcd 397.9520, obsd 397.9462.

To a solution of 160 mg (0.40 mmol) of 6-(*N*-tetrachlorophthalimide)caproic acid and 121 mg (1.20 mmol) of triethylamine in 25 mL of dichloromethane was added 205 mg (0.80 mmol) of *N*,*N*′-disuccinimidyl carbonate and the mixture stirred for 2 h under argon at room temperature. The succinimidyl ester was recovered by evaporation of solvent. Purification (silica gel, elution with 1:1 ethyl acetate/dichloromethane) gave 170 mg (85%). ^{1}H-NMR: (DMSO-d_6, 300 MHz) δ 3.56 (t, 2H, J = 6.9 Hz), 2.78 (s, 4H), 2.65 (t, 2H, J = 7.2 Hz), 1.69–1.56 (m, 4H), 1.41–1.31 (m, 2H). ^{13}C-NMR: (DMSO-d_6, 500 MHz) δ 170.2, 168.9, 163.5, 138.0, 128.5, 127.9, 37.9, 30.0, 27.0, 25.4, 25.1, 23.8. MS m/z (LSIMS$^+$): calcd 494.9684, obsd 494.9695.

The succinimidyl ester (52 mg, 105 μmol) was dissolved in 0.80 mL of 1,4-dioxane, and 0.60 mL (76 μmol) of the resulting solution was added to 10 mg (0.15 μmol) of BSA in 5 mL of 100 mM $NaHCO_3$(aq), pH 8.0. In a parallel experiment, while 0.12 mL (15 μmol) of the ester solution was added to 10 mg (15 nmol) of thyroglobulin (TG) in 3 mL of 100 mM $NaHCO_3$(aq), pH 8.0. After 16 h at room temperature, the BSA and TG conjugates were purified by size exclusion chromatography (G-50 column) using PBS-7.5 (10 mM phosphate, 140 mM NaCl, pH 7.5). Amine titration[13] indicated 44 haptens/BSA molecule and 121 haptens/TG molecule.

Antibody Production. Mice (129 GIX$^+$ strain) were immunized with the TG conjugate of 6-(*N*-tetrachlorophthalimide)caproic acid emulsified in MPL + TDM emulsion (RIBI ImmunoChem Research, Inc.). Serum titer was determined with the BSA conjugate **4** by ELISA.[7,8] Hybridomas were prepared by fusion of SP2/0+ myeloma cells with the spleen cells of a hyperimmunized mouse.[14] Cell lines secreting IgG antibodies specific for the hapten were subcloned twice, and the four clones with the highest titers were propagated in mouse ascites using (BALB/c × 129 GIX$^+$)F_1 mice.[14] Monoclonal antibodies were purified from ascites fluid by ammonium sulfate precipitation followed by cation exchange chromatography (DE52 from Whatman). Protein was eluted from the gel with a NaCl step gradient (0, 50, 100, 150, 250, 500 mM) in 10 mM Tris·HCl (pH 8.0). IgG was eluted at 100 mM NaCl, concentrated, and dialyzed exhaustively against MBS-6.0. Protein concentration was determined by ultraviolet absorption at 280 nm assuming a molar extinction coefficient, ϵ_{280}, of 220 000 M^{-1} cm^{-1} and a molar mass of 160 000 g mol^{-1}.[7] The affinities of the purified antibodies for product **4** and substrate **2** were evaluated by ELISA, and the antibody 11A5 was identified as exhibiting the best discrimination between

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substrate and product. The antibody 11A5 was covalently linked to horseradish peroxidase (HRPO) using ImmunoPure activated peroxidase from Pierce. The conjugate (11A5-HRPO) was stored at −20 °C (long term) or 4 °C (short term).

CatELISA. Microtiter plates (96-well A/2 Costar ELISA plates) were coated with BSA–maleimide conjugate **2** (50 μL, 15 μg/mL in MBS-6.0) for 1 h at 37 °C, shaken dry, and blocked with BSA (100 μL, 3% in MBS-6.0) for 1 h at 37 °C. The plates were then rinsed with distilled water (dH_2O) and shaken dry. When the concentration of catalytic antibody 1E9 was varied, 25 μL of MBS-6.0 was added to each well, and a stock of 10 μM 1E9 in MBS-6.0 was serially diluted 1 in 2 across the plate. Then, a solution (25 μL) of TCTD in MBS-6.0 (6.7% CH_3CN) was added to each well and mixed by pipetting up and down several times. The reaction was left to proceed for 1 h at room temperature. When 1E9 concentration was kept constant and reaction time varied, all wells on the plate were filled with MBS-6.0 and the reaction was initiated at various times by replacing the buffer with a solution (50 μL) containing TCTD and 100 nM 1E9 in MBS-6.0 (3.3% CH_3CN). Following the reaction, the plates were rinsed thoroughly with dH_2O and incubated for 1 h at 37 °C with 11A5-HRPO diluted in binding buffer (10 mM phosphate, 2 M NaCl, 40 mM $MgSO_4$, 0.05% Tween-20). The concentration of active 11A5-HRPO used for this incubation was determined empirically (see text). The plates were rinsed extensively with dH_2O and developer was added (40 μL of 0.3 mg/mL 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS), 0.02% H_2O_2 in 0.1 M citrate, pH 4.0). Absorbance at 405 nm was recorded after about 30 min and the average absorbance (≈0.08) of several control wells (no TCTD during reaction incubation) subtracted.

Estimation of Dissociation Constants. Standard ELISAs[7,8] were performed by immobilizing antigen and titrating purified antibody by serial dilution. Bound antibody was detected using goat anti-mouse kappa-HRPO (FisherBiotech). Absorbance at 405 nm was recorded following color development. Since the absorbance is proportional to the fraction of antigen bound by antibody, the dissociation constant K_d was determined by fitting the data to the equation

$$A_{405nm} = c\frac{[Ag]_t + [Ab]_t + K_d - (([Ag]_t + [Ab]_t + K_d)^2 - 4[Ag]_t[Ab]_t)^{1/2}}{2[Ag]_t}$$

where c is the constant of proportionality, $[Ag]_t$ is the total antigen concentration, and $[Ag]_t$ is the total antibody concentration. The parameters c and K_d were abstracted by general curve-fitting techniques.[15] Analysis of this equation reveals that the estimate of K_d does not change significantly with $[Ag]_t$ if $[Ag]_t \ll K_d$. This regime was maintained for all determinations.

Results and Discussion

The strategy of the catELISA for a bimolecular reaction is outlined in Figure 1. Immobilizing one of the substrates on the surface of a 96-well microtiter plate can be achieved by covalently linking it to BSA, which adheres strongly to polystyrene surfaces. The choice of substrate to immobilize and the site of linker attachment is dictated by a need to maximize the antigenic distinction between the substrate and product while avoiding interference with the reaction and maintaining synthetic accessibility. Considering these criteria for the Diels–Alder reaction of Figure 2, the maleimide–protein conjugate **2** was synthesized. The phthalimide product of the reaction, **4**, was also prepared, and protein conjugates of the compound were used to elicit a product-specific monoclonal antibody (11A5). Horse radish peroxidase was covalently linked to 11A5 to yield the enzyme-labeled antibody, 11A5-HRPO.[16]

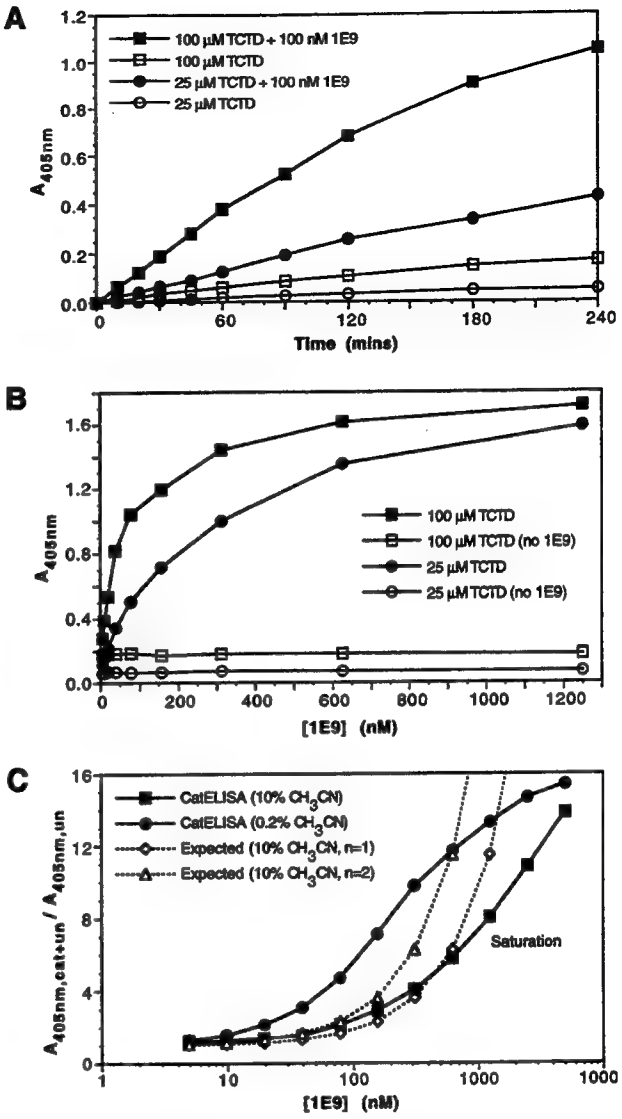

Figure 3. catELISA results. Each of the three experiments were performed separately; hence, the absolute absorbance values cannot be compared between experiments. In each case, the mean absorbance of several control wells in which TCTD was not added was subtracted: (A) dependence of the signal (A_{405nm}) on the length of the reaction; (B) dependence of the signal (A_{405nm}) on the concentration of 1E9 (antibody catalyst); (C) dependence of the ratio $A_{405nm,cat+un}/A_{405nm,un}$ on the concentration of 1E9. Experimental results from the catELISA performed with 10% acetonitrile and 0.2% acetonitrile are shown with solid lines. The expected results, based on published data,[9] for the reaction in 10% acetonitrile are shown with dashed lines. Expected results were calculated for $n = 1$ and $n = 2$ (see text), representing the upper and lower bounds of $A_{405nm,cat+un}/A_{405nm,un}$.

The catELISA was assessed by studying the time dependence of the reaction between tetrachlorothiophene dioxide (**1**, TCTD) and immobilized maleimide **2** in the presence or absence of antibody catalyst 1E9 (Figure 3A). The antibody 1E9, raised against the transition state analog **5** (TG conjugate), has previously been shown to catalyze the reaction of *N*-ethylmaleimide (**2**, *R* = ethyl) with TCTD in solution.[9] The signal (A_{405nm}), propor-

(15) KaleidaGraph (Abelbeck Software).

(16) The product can also be recognized by unmodified 11A5, which is detected in a subsequent step by a commercially available enzyme-linked goat antibody which recognizes the constant region of mouse antibody kappa chains. The direct linking of horse radish peroxidase to the detection antibody 11A5 increases the convenience of the assay by reducing the number of steps. Furthermore, since the catalytic antibody 1E9 is also a mouse antibody, direct detection decreases the "noise" in the data that is caused by small amounts of 1E9 bound to the plate.

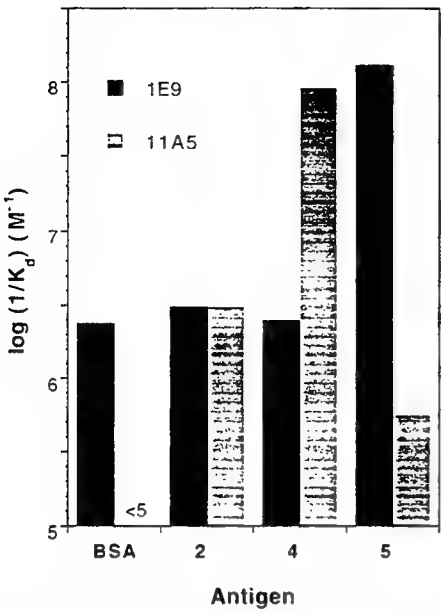

Figure 4. Affinities of the antibodies 1E9 and 11A5 for various antigens. The dissociation constants were determined as described in the Materials and Methods section.

tional to the amount of product **4** formed during the reaction, increased linearly with time for at least the first hour of the reaction, leveling off at longer reaction times. The addition of 1E9 resulted in an increased signal, whereas addition of non-catalytic antibodies had no discernible effect (data not shown). Furthermore, increasing the concentration of TCTD resulted in an appropriately higher signal. The dependence of the signal on catalyst concentration was also followed at different TCTD concentrations, maintaining a reaction time of 1 h (Figure 3B). The signal appeared to increase linearly with [1E9] at low concentrations, leveling off at higher concentrations. Again, the signal was appropriately larger with higher [TCTD].

Formation of product is expected to slow down as the reaction proceeds. However, the leveling off observed at long reaction times and high catalyst concentrations (Figure 3A,B) is not believed to arise from substrate depletion. Previously determined kinetic parameters for 1E9[9] predict the extent of the reaction to be too small under these conditions to account for the observed effect. Rather, it is believed that the leveling off results primarily from saturation of 11A5-HRPO binding, since the density of substrate or product moieties on a BSA molecule exceeds the physically attainable density of detection antibody due to size constraints. The affinity of 1E9 for BSA (Figure 4) may also contribute to the saturation phenomenon: the rate of the catalyzed reaction will cease to increase linearly when [1E9] approaches or exceeds the apparent dissociation constant of 1E9 for BSA (420 nM). It is important to note that the control afforded by varying both the reaction time and TCTD concentration enables saturation to be avoided, thereby maximizing the signal to background ratio at a given concentration of catalyst.

The accuracy of the catELISA can be assessed by deriving kinetic parameters from the results and comparing them to previously determined values. However, since the concentration of product cannot be determined explicitly and since saturation arises from factors not easily related to the extent of the reaction, the absolute rates of the catalyzed or uncatalyzed reactions cannot be determined from the data. Nevertheless, the rate of the catalyzed reaction *relative* to the uncatalyzed reaction can be determined. The signal (A_{405nm}) is proportional to the concentration of product formed during the course of the reaction. This can be described by the integrated rate law for a first-order rate equation since the reaction occurs under pseudo-first-order conditions (TCTD is present in large excess) and the concentrations of both substrates are maintained well below their K_m values. Thus,

$$\frac{A_{405nm,cat+un}}{A_{405nm,un}} = \frac{1 - \exp(-n(k_{cat}/K_{m(2)})[1E9]t - k_{un}[TCTD]t)}{1 - \exp(-k_{un}[TCTD]t)} \quad (1)$$

Here, $A_{405nm,cat+un}$ and $A_{405nm,un}$ are the signals observed in the presence and absence of added catalyst 1E9, $k_{cat}/K_{m(2)}$ is the specificity constant of 1E9, k_{un} is the second-order rate constant for the uncatalyzed reaction, and n is the number of effective active sites of the antibody IgG molecule. Although the antibody IgG molecule has two active sites, if the density of the BSA–substrate conjugate on the plate is extremely low, only one site can participate in catalysis at a given time and $n = 1$. If, on the other hand, the density is very high, both antibody binding sites may have access to substrate at all times and $n = 2$. Between these two extremes, $1 < n < 2$.

The ratio $A_{405nm,cat+un}/A_{405nm,un}$ was calculated from catELISA data covering a large range of catalyst concentrations, and the expected values were calculated from published data[9] using eq 1. Since n, the number of effective active sites on the antibody molecule, cannot be determined experimentally, expected values were calculated for $n = 1$ and $n = 2$ and the resulting upper and lower bounds plotted with the catELISA data (Figure 3C). The results from the catELISA, performed under the same conditions used to determine the published data, fall within the expected bounds at low 1E9 concentrations, exhibiting saturation at higher concentrations. In the original studies,[9] 10% acetonitrile was included to increase the solubility of TCTD in the aqueous buffer. Since the detection limit of the catELISA permits much lower concentrations of TCTD, the assay was also performed in 0.2% acetonitrile for comparison. If the value of k_{un} for the reaction in solution is used to calculate the corresponding value of $k_{cat}/K_{m(2)}$ by general curve fitting[15] of the catELISA data to eq 1, the apparent $k_{cat}/K_{m(2)}$ at 25 μM TCTD and 10% acetonitrile falls in the range 6.5–13 M^{-1} min^{-1} (bounds determined by $n = 1$ and $n = 2$). This compares to an expected value of 8.4 M^{-1} min^{-1}, calculated from the published data.[9] At 0.2% acetonitrile, $k_{cat}/K_{m(2)}$ falls in the range 30–59 M^{-1} min^{-1}, consistent with observations that 1E9 activity is increased at lower acetonitrile concentrations (K. Hill, unpublished data).

Substrate(s). In developing a screening procedure such as this, it is necessary to consider the requirements and limitations of each element of the assay. The Diels–Alder reaction studied here provides a representative system for evaluating the various features involved and some of the potential complications that must be addressed.

One problem that arises is that of substrate instability. For the reation under study, both substrates present complications. The diene, TCTD, is particularly subject to Michael addition, and buffers containing nucleophiles (such as Tris) must be avoided. Besides buffer molecules, nucleophilic groups on the catalyst and on the BSA molecules, such as the ϵ-amino groups of surface-accessible lysine residues, must be considered. Reaction of TCTD with these proteins can lead to reduction of TCTD concentrations, destruction of enzyme activity by reaction with residues near the active site, or cross-linking of detection antibody to BSA on the surface of the plate, yielding false positive signals. In the original characterization of 1E9,[9] such complications were avoided by reductive methylation of the amines on 1E9 and by reducing the pH to 6.0. In the catELISA assay, reducing the pH to 6.0 minimized these side reactions sufficiently to obtain reproducible signals so that modification of amines was not required. Although cross-linking of detection antibody to the plate becomes problematic at higher pH, this side reaction can be blocked by

acetylation of the BSA coating the plate with acetic anhydride (data not shown).

The maleimide substrate **2** is also susceptible to undesired side reactions: Michael addition of nucleophiles to the double bond and ring-opening by hydrolysis[12] compete with the Diels–Alder reaction. The half-life of **2** under the experimental conditions can be determined conveniently by catELISA, varying the time it is preincubated with buffer prior to reaction with TCTD. Even when the pH is reduced to 6.0, the half-life of the BSA–maleimide conjugate is under 5 h at room temperature in MES buffer. Nevertheless, we find that these competing reactions are effectively independent of catalyst concentration so that the signal to background ratio is unaffected by the instability of **2**.

Detection Antibody. Fundamental to any reaction assay is the ability to distinguish product from substrate. In the catELISA this is achieved through the remarkable binding specificity of antibody molecules. The *degree* of discrimination between product and substrate can be defined by considering a hypothetical experiment in which product and substrate are immobilized in separate wells of a microtiter plate at the same concentration. The detection antibody is then added to each well and allowed to bind. The ratio of the fraction of product bound by antibody to the fraction of substrate bound by antibody, $F_{p/s}$, provides a good index of discrimination between product and substrate. From standard equilibrium equations, we know that the fraction of antigen bound by an antibody depends on the total antigen concentration ($[Ag]_t$), the total antibody concentration ($[Ab]_t$), and the dissociation constant of the antibody for the antigen ($K_{d,Ag}$). Thus, an expression for $F_{p/s}$ as a function of these three parameters can be derived:

$$F_{p/s} = \frac{[Ab]_t + [Ag]_t + K_{d,p} - (([Ab]_t + [Ag]_t + K_{d,p})^2 - 4[Ab]_t[Ag]_2)^{1/2}}{[Ab]_t + [Ag]_t + K_{d,s} - (([Ab]_t + [Ag]_t + K_{d,s})^2 - 4[Ab]_t[Ag]_t)^{1/2}} \quad (2)$$

Here, $K_{d,p}$ and $K_{d,s}$ are the dissociation constants of the detection antibody for the product and substrate, respectively. Analysis of eq 2 reveals that the maximum value of $F_{p/s}$ is $K_{d,s}/K_{d,p}$, approached as $[Ag]_t$ and $[Ab]_t$ both tend to 0 (a derivation of this statement is provided as supplementary material). Furthermore, to achieve >85% of the maximum discrimination, both $[Ag]_t$ and $[Ab]_t$ must be less than 10% of $K_{d,p}$. This is an extremely important aspect to consider when determining the conditions of the assay. For example, if the concentration of antibody used to detect the product is too high (e.g., close to $K_{d,s}$), very little discrimination between product and substrate will be observed, even if $K_{d,p} \ll K_{d,s}$. In practice, $[Ag]_t$ and $[Ab]_t$ can be optimized empirically.

Assuming $[Ag]_t$ and $[Ab]_t$ are chosen such that $F_{p/s} \approx K_{d,s}/K_{d,p}$, the ability to identify catalysts in a library of variants depends on both $K_{d,s}/K_{d,p}$ and the *extent* of the reaction. Since confident identification is limited by the precision of the data (estimated to be about 10% for enzyme immunoassays),[8] the signal resulting from product binding must be >10% of the background resulting from substrate binding. If the detection antibody offers only a small value of $K_{d,s}/K_{d,p}$, longer reaction times will be needed to increase the extent of the reaction. Large values of $K_{d,s}/K_{d,p}$ obviate the need for longer reaction times. For the reaction studied in this paper, long reaction times were inaccessible due to substrate instability. Thus, an antibody with a large value of $K_{d,s}/K_{d,p}$ was required. Dissociation constants of 1E9 and 11A5 were estimated for substrate **2** and product **4**, as well as for BSA and for the transition state analog **5** (see Figure 4). The apparent values of $K_{d,s}$ = 320 nM and $K_{d,p}$ = 11 nM yield $F_{p/s,max}$ = 29. This corresponds well to the observed $F_{p/s}$ of the 11A5-HRPO conjugate under the conditions of the assay and was more than sufficient to permit unequivocal detection of catalysis.

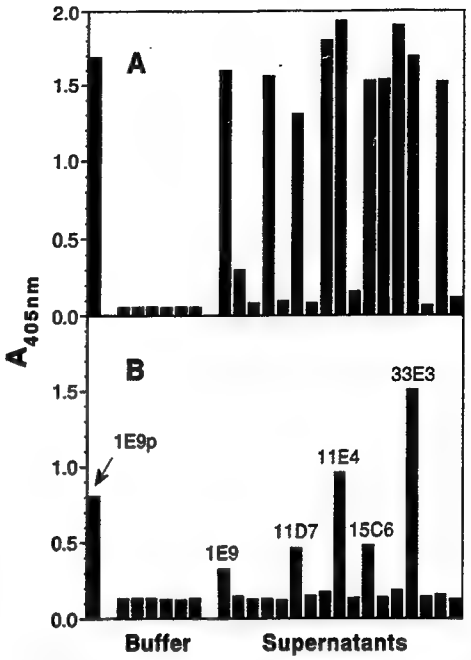

Figure 5. (A) Standard ELISA results with immobilized transition state analog **5**: lane 1, 100 nM purified 1E9; lanes 3–8, MBS-6.0 buffer (negative controls); lanes 10–26, hybridoma supernatants, diluted 50% with binding buffer. Bound antibody was detected with a commercial goat anti-mouse antibody covalently linked to horseradish peroxidase. (B): catELISA results with immobilized substrate **2**. The reaction was initiated by diluting the samples 50% with TCTD in 60 mM acetate + 40 mM NaCl (pH 5.0), giving a final concentration of 100 μM TCTD. The reaction was allowed to proceed for 1 h at room temperature. Product was subsequently detected with 11A5-HRPO. The lanes of part B correspond to those of part A. Hybridoma supernatants displaying significant catalytic activity are labeled.

The high value of $F_{p/s}$ in our system was obtained by using the monoclonal antibody 11A5. Since production of monoclonal antibodies is time consuming and costly, it would be advantageous, when possible, to use polyclonal serum to detect the product. This has proved sufficient for at least one reaction.[6] However, we have found that the use of polyclonal serum is often not possible. If the substrate and product have strongly antigenic elements in common, polyclonal serum is likely to contain antibodies that recognize these common functionalities. We have shown for a phosphate ester hydrolysis reaction involving a phosphotyrosine-containing pentapeptide substrate that polyclonal serum offered no detectable discrimination between substrate and product (J. Shin, unpublished). The observed $F_{p/s}$ of the serum was increased somewhat by passing the serum through a column of excess immobilized substrate. However, even after this purification step, the value of $F_{p/s}$ was less than 2, seriously compromising the reliability of the assay.

Utility of the catELISA. The data of Figure 3 demonstrate the successful application of the catELISA to a bimolecular, cycloaddition reaction. Our results, together with those of Tawfik *et al.*,[6] support the potential of this assay as a general approach to screening for catalysts, complementing and extending other strategies reported to date.[3,5] In our example, control over the extent of the reaction is achieved by varying both the time of the reaction and the concentration of the second substrate added in solution, enabling conditions to be chosen that optimize the signal to background ratio by avoiding saturation of product formation or detection. Under nonsaturating conditions, the catELISA provides a remarkably accurate estimate of the efficiency of the catalyst being assayed, despite immobilization of one of the substrates.

Applying these results, the catELISA was used to screen hybridoma supernatants from the original fusion that yielded

1E9. Supernatants were tested for the ability to bind the transition state analog **5** by regular ELISA (Figure 5A) and for the ability to catalyze the Diels–Alder reaction of Figure 2 by catELISA (Figure 5B). The results of Figure 5B clearly demonstrate that the catalytic antibody 1E9 can be distinguished from other, noncatalytic antibodies and from negative controls (no antibody) by catELISA. Higher signals over background have been noted for other samples of 1E9 supernatant, likely reflecting higher antibody concentrations in these samples. Interestingly, four new catalysts were identified, each displaying significant catalytic activity (Figure 5B). All four of these new catalysts bind the transition state analog **5** (Figure 5A), consistent with the strategy used to elicit the catalysts. Notably, the previously untested 33E3 is substantially more active than 1E9 at comparable concentrations, as estimated by ELISA titration. Characterization of these antibodies is currently in progress.

The success of this screen can be attributed to the remarkably low limit of catalytic activity that can be identified unambiguously by catELISA. Two factors affect this limit: the precision of the data and the minimum amount of product that can be detected. Estimating the precision to be about 10%,[8] the rate of the catalyzed reaction could in principle be as low as 10% of the rate of the background reaction and still allow identification of a catalyst. If the background reaction is extremely slow, product detection may instead define the lower limit of catalytic activity that can be identified. However, 10–0.1 fmol of antigen can often be detected in a microtiter plate well by enzyme immunoassay, depending on the affinity of the antibody for the antigen.[8] This exquisite level of detection means that in most cases, including the reaction under study, it is the precision of the data that determines the level of activity that can be observed. Since catalytic activity is a function of both the efficiency and concentration of the catalyst, high concentrations of the species being assayed enable relatively inefficient catalysts to be found, while lower concentrations result in more restricted searches. In the case of the Diels–Alder reaction, the data of Figure 3 predict that catalysts as efficient as 1E9 can, in principle, be detected at concentrations as low as 2 nM. In practice, other factors increase the variation in the data, and a more realistic lower limit for 1E9 would be about 10 nM. Less efficient catalysts would require higher concentrations, while more efficient catalysts could be detected at even lower concentrations.

The ability to detect catalysts at such low concentrations has important practical implications for screening large libraries of variants. As demonstrated in Figure 5B, catalytic activity can be identified in hybridoma supernatants, where antibodies are typically expressed at concentrations of 30–300 nM.[7] However, it should also be possible to exploit the catELISA for identification of highly active catalysts in libraries of Fab molecules selected using phage display methods[17] and expressed in microorganisms. This technology lends access to much larger sizes of libraries, increasing the probability of finding more active catalysts. Furthermore, the catELISA is not restricted to screening antibodies. Other proteins or even nonproteinaceous catalysts might be identified using this powerful approach. We are currently using the assay described in this paper to screen libraries of Fab molecules, single-chain Fv species, and randomized oligoribonucleotides.

Acknowledgment. Partial support of this work by the National Institutes of Health (GM38273), the Army Research Office, and the Office of Naval Research is gratefully acknowledged. G.M. is the recipient of a Natural Science and Engineering Research Council of Canada 1967 Centennial Postgraduate Scholarship. We also thank Theodore Tarasow for useful suggestions and Richard Daniels for the hybridoma work.

Supplementary Material Available: Derivation of eq 2 and limit of $F_{p/s}$ as $[Ab]_t$ and $[Ag]_t$ approach 0 (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(17) Chiswell, D. J.; McCafferty, J. *TIBTECH* **1992**, *10*, 80.

Enrichment for RNA molecules that bind a Diels-Alder transition state analog

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ABSTRACT RNA molecules that bind a transition state analog for a Diels-Alder reaction (K_d=0.35 ± 0.05 mM) were isolated from a starting pool of approximately 10^{14} sequences by affinity chromatography. After the initial rise and plateau of the amount of RNA that eluted with soluble analog, a step gradient elution was used to further enrich the pool for sequences with higher affinities for the target. This is the first report of the isolation of RNA molecules that bind either a non-planar or a hydrophobic ligand. A conserved nucleotide sequence and secondary structure present in many of the RNA molecules is necessary but not sufficient for binding the analog. No catalysts of the targeted Diels-Alder reaction were found among the binders. The absence of catalysis contrasts with previous successful experiments with antibodies and suggests that other strategies may be needed to identify oligonucleotides with diverse catalytic activities.

Theories of evolution based on RNA as the progenitor molecule were fueled by the discovery of RNA molecules capable of catalyzing transesterification reactions (1, 2). Recently, a derivative of the *Tetrahymena* ribozyme was shown to hydrolyze an aminoacyl ester bond with a modest rate acceleration (3), and there is strong evidence that the peptidyl transferase activity of the ribosome is largely due to RNA (4). In addition, ribozymes with novel catalytic properties have been isolated from pools of mutagenized *Tetrahymena* self-splicing introns (5-7) and tRNAs (8, 9) and from pools of random RNA molecules (10). However, in an "RNA world" these primitive oligonucleotide catalysts must have been able to interact with a variety of substrates and to accelerate a wide range of chemical transformations.

RNA molecules that bind a variety of proteins (11-16) and small molecules (17-21) have been isolated from large pools of random sequences through a reiterative selection and amplification process called SELEX (Systematic Evolution of Ligands by EXponential enrichment) (11). We are seeking to utilize this technology to develop a general scheme for surveying the catalytic repertoire of RNA and to examine the variety of ligands that can be specifically bound by nucleic acids. Antibodies that catalyze an extensive list of chemical reactions have been elicited by transition state analogs (22-24). Similarly, RNAs that tightly bind such analogs might also catalyze the corresponding reactions.

Here, we report the isolation of RNA molecules that bind a transition state analog for a Diels-Alder reaction (Fig. 1). This

analog successfully elicited antibodies capable of catalyzing the cycloaddition of tetrachlorothiophene dioxide (TCTD) with *N*-ethylmaleimide (NEM) (25). This target was chosen for three reasons: 1) the bimolecular Diels-Alder reaction is an excellent candidate for RNA catalysis since the condensation of the substrates is strongly driven by proximity effects, and the large conformational changes that accompany the reaction can be exploited to minimize problems due to product inhibition; 2) a catalytic antibody elicited by this analog is available for direct comparison of the catalytic efficiency of protein and any RNA isolated; and 3) it tests the limits of RNA-ligand recognition by demanding that the nucleic acid bind a molecule that is non-planar and hydrophobic.

MATERIALS AND METHODS

Target Synthesis. The *N*-hydroxysuccinimidyl ester of the transition state analog was prepared as previously described (25). 12 ml preswollen Sepharose EAH (Pharmacia) was washed with 1 L 0.5 M NaCl and then equilibrated with 1:1 Dioxane/100 mM $NaHCO_3$ (pH 8.0). The activated ester was dissolved in 1.5 ml of dioxane and added to the resin at room temperature. The resulting slurry was incubated at 5 ^oC for 16 hr with occasional agitation. Remaining free amines were acylated by treatment of the resin with 1 ml of acetic anhydride at 5 ^oC for 2 hr. The resin was then washed with 150 ml of 1:1 dioxane/100 mM $NaHCO_3$ (pH 8.0) followed by 500 ml of PBS (pH 7.5).

SELEX. The random RNA molecules for selection were transcribed *in vitro* from a double-stranded DNA template containing a random region 80 nucleotides long flanked by regions with fixed sequences as previously described (11). 1 nmol unlabeled RNA and 500 Kcpm radioactively labeled RNA were denatured at 70 ^oC for 5 min in 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl. The $MgCl_2$ concentration was adjusted to 5 mM and the sample was immediately incubated at 0 ^oC for 10 min. The RNA solution was allowed to equilibrate to room temperature and applied to 0.1 ml of the derivatizeditized Sepharose EAH which had been prewashed with 1 ml of wash buffer (50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM $MgCl_2$). The RNA was incubated with the column matrix for 15 min before washing with 1 ml of wash buffer. Bound RNA was eluted with wash buffer containing various concentrations of soluble transition state analog (see results). A cDNA copy of the

eluted RNA molecules was synthesized with AMV reverse transcriptase and amplified by PCR using standard methods (11).

The binding affinities of selected RNA molecules were determined by analytical affinity chromatography (19) with 1 mM and 10 mM soluble analog.

Cloning and Nucleotide Sequence Determination. Individual molecules were isolated from selected pools by ligating *Bam*HI and *Hin*dIII digested PCR products into pUC18. The ligated DNA was introduced into *E. coli* strain SURE (Stratagene) by electroporation, plasmids were isolated, and the nucleotide sequences in the inserted DNAs were determined by standard dideoxynucleotide methods. The sequences were searched for patterns in their primary structures (gap penalty of 2) (26) and in their possible secondary structures (27, 28).

Truncated RNAs. RNA molecules containing only the consensus secondary structure were transcribed from synthetic DNA templates whose complementary strand had been made using standard PCR techniques. Deletions were introduced into the 15-B-9 and 15-B-11 PCR templates using primers that hybridized within the double-stranded DNA. The transcription products were purified, applied to the derivatized column, and eluted with 1 ml of 50 mM transition state analog as described above. Each RNA was tested in at least two independent trials.

Assays for Enzymatic Activity.

The starch/iodide assay was conducted in 96-well microtiter plates containing 100 µl/well of 10 µM RNA, 500 µM TCTD, 5 mM NEM, 250 µM I_2, 0.084% starch, 5 mM $MgCl_2$, 0.5 M NaCl, 50 mM Tris-HCl (pH 7.5)

and 3% MeCN. The reactions were monitored at 605 nm with a microtiter plate reader (Molecular Devices) at room temperature for at least 1 hr. The antibody 1E9 (24) was used as a positive control.

The catELISA assay was carried out as previously described (30, 31).

RESULTS

Selection of RNA Molecules that Bind a Transition State Analog. RNA molecules that bind to the hexachloronorbornene derivative **1** (Fig. 1) were isolated from a pool of approximately 10^{14} sequences containing 80 random nucleotides flanked by 25 base fixed regions. The RNA pool was folded in 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl and 5 mM $MgCl_2$ and applied to a Sepharose column to which the analog was covalently attached at a concentration of approximately 10 mM. The column was washed extensively before bound RNA molecules were eluted with 50 mM soluble analog. cDNA copies of the eluted RNA molecules were synthesized by reverse transcriptase and amplified by PCR. The PCR product was used as template for transcription of RNA for the next round of selection.

In the first six rounds, less than 1% of the RNA molecules bound to the column. Binding increased to 2.9% in round 7 and 20% in round 8. Two additional rounds of selection and amplification did not significantly increase the amount of RNA bound to the column. Moreover, reapplication of the round 9 RNA that was not retained by the column again resulted in the same percent binding (21%) indicating that the maximum degree of enrichment attainable by this method had been reached.

Analysis of the nucleotide sequence in 125 clones from the round 9 pool showed that it still consisted of a complex, but not random, mixture. Twelve sequences were repeated one or more times. In addition, a conserved sequence appeared to be present at the 5' end of the random region in eleven of the molecules (see below).

Rounds of selection using concentration gradient elutions were used to further decrease the complexity of the selected RNA pool. In round 11, a step gradient of four column volumes each of 5 mM, 10 mM, 25 mM and finally 50 mM analog was used to elute bound RNA from the column. RNAs with the weakest affinity for the column-bound analog elute with the lowest concentrations of soluble analog, whereas the higher affinity RNAs require increased concentrations of ligand for elution. RNA molecules that eluted in the 5 mM analog fractions (population A) and 50 mM analog fractions (population B) were collected and amplified. Starting with round 12, population A was applied to the column and eluted with 5 mM analog. In a separate experiment, population B was eluted with the four-step gradient, and those RNAs that dissociated from the column at the highest concentration of analog (50 mM) were collected. Over five rounds of selection and amplification, the elution profile and the total amount of RNA eluting from the column changed according to which part of the gradient was collected and amplified for the next round of selection (Fig. 2). In round 11, 26% of the RNA bound to the column and eluted with a bell shaped profile. By round 15, the total amount of population A RNA that bound to the column remained unchanged (27%), but the majority eluted with the lowest concentrations of analog. In contrast, the total amount of population B RNA that bound to the column dramatically increased to 41% by round 15 and required higher concentrations of the analog for elution.

Various gradients were used in an attempt to further purify the tightest binding RNA species from the round 15-B pool. However,

four rounds using a two step gradient of 25 mM and 50 mM analog and a fifth round using a third step of 84 mM analog elution did not have an effect on the amount of RNA that bound to the column. A determination of the nucleotide sequences of members of several pools (15-B, 19-B and 20-B) showed that the gradient elution process had significantly narrowed the complexity from that of the round 9 pool. There were only 82 unique sequences among 153 clones. However, no single sequence dominated the pool.

A Conserved Sequence. Analysis of the sequence of nucleotides in RNA molecules (26) from the round 9, round 15-B, round 19-B and round 20-B pools led to the identification of a 21 nucleotide conserved sequence (Fig. 3A) that was present in 10% of the 113 unique round 9 RNAs and in 29% of the 82 unique sequences from later rounds. In all but one case, this sequence occurred at the 5' end of the random region of the RNAs. No sequence homology was found downstream of the conserved element. Folding of these RNA sequences by a computer algorithm (27, 28) consistently yielded a bulged stem-loop structure formed by interaction of the seven nucleotides at the 3' end of the consensus sequence with the 5' end fixed region (Fig. 3B). Strong evidence for this structure was provided by a clone from round 15-B (15-B-9) which had a portion of the consensus sequence and was capable of forming the same secondary structure entirely from nucleotides in its random region (Fig. 3C). The stem is composed of different base pairs in 15-B-9 providing covariational evidence for the helix. Surprisingly, even the sequence of the bulge contributed by the fixed sequence in the other RNAs is conserved in 15-B-9, apart from the addition of a

single U. An additional G-U base pair leaving a bulged C in the top stem would make the large bulge in 15-B-9 even more similar to the consensus structure (Fig. 3C).

Deletions were introduced into the template encoding 15-B-9 and into a clone with the consensus structure at the 5' end of the molecule (15-B-11) (Fig. 4). The 15-B-9 RNA lacking 35 nucleotides from its 3'-end [15-B-9(95-129)] bound to the column slightly better than the full length molecule. This deleted region may either destabilize the structure of the full length RNA or interfere with its recognition of the analog. All of the truncated RNAs in which the sequence of the consensus structure was intact retained at least modest capacity (>10% of the full length RNA) to bind to the column, whereas those in which the sequence was disrupted no longer bound the analog (<1% of the full length RNA). In a second experiment, an RNA containing the consensus sequence and the ability to form an eight base pair helix (Fig. 3D) was transcribed from a synthetic DNA template and applied to the column. This 38 nucleotide RNA was not retained by the column-bound analog, nor was a similar RNA in which the helix was extended by an additional six base pairs. Therefore, the consensus sequence is essential but not sufficient for RNA binding to the column.

Determination of Binding Affinities. The affinity of the selected RNA molecules for the hexachloronorbornene derivative **1** in solution was determined by analytical affinity chromatography (19). The mean K_d for the round 15-A and round 15-B pools was measured to be 1.5 ±0.5 mM and 0.45 ±0.05 mM respectively, and that

of the 15-B-11 clone, which has the conserved sequence at its 5'-end, was determined to be 0.35 ±0.05 mM.

Screening for Catalysts. Two methods were used to assess the ability of the RNA molecules that bind the transition state analog to catalyze the Diels-Alder reaction. In the first assay, the bleaching of a starch/iodide solution by the SO_2 liberated following the initial cycloaddition of the substrates is monitored spectroscopically. This method was abandoned after 117 round 9 RNA clones were screened because of its lack of sensitivity due to the low solubility of starch under the reaction conditions. A more sensitive catELISA assay (30, 31) was developed and used to screen 18 unique clones from round 15-B, 50 unique clones from round 19-B and 15 unique clones from round 20-B. In addition, 64 clones from round 9 were re-screened for catalytic activity by this method. In all, 196 unique clones were analyzed by one, or both, of the methods without the identification of an RNA molecule that could accelerate the rate of the Diels-Alder reaction.

DISCUSSION

RNA molecules that bind a transition state analog of a Diels-Alder reaction were isolated from a starting pool of approximately 10^{14} sequences. After the initial rise and plateau of the amount of RNA retained by the column, the use of a step gradient elution further enriched the pool for RNAs with higher affinities for the target. Variations of this technique could be used to isolate nucleic acids with enhanced affinity for any target from large pools of sequences.

This is the first report of the isolation of RNA molecules that bind either a non-planar or a hydrophobic ligand. Other reports have involved ligands that are planar and/or positively charged (17-21). RNA presumably binds the later molecules through base stacking interactions and favorable charge-charge contacts with the phosphate backbone. RNA is apparently capable of forming a relatively hydrophobic binding pocket that complements the non-planar topology of the hexachloronorbornene derivative **1** and may take advantage of the few hydrogen bonding opportunities presented by the imide functionality. A fairly large number of very different RNA sequences appear to form structures capable of binding this molecule.

The conserved nucleotide sequence present in many of the selected RNA molecules (Fig. 3) provides a starting point for examining how RNA can bind this unusual ligand. The proposed secondary structure formed by this conserved sequence and the 5' fixed region is strongly supported by its occurrence entirely within the random region of a clone. The step gradient elution enriched the consensus

in the population, suggesting that this sequence contributes to the structure of the RNA molecules with the highest affinities for the analog. The K_d of one of these RNAs, 15-B-11, (0.35 ±0.05 mM) is lower than the mean of the round 15-B pool (0.45 ±0.05 mM). The nucleotide deletion experiments demonstrate that the region of the RNA that contains the structure is essential for binding the analog. However, the conserved sequence alone is not retained by the column. It may require other sequence elements to fold into the correct conformation or to bind the target.

It is discouraging that none of the 196 unique RNA molecules had any detectable catalytic activity. This transition state analog elicited an antibody (1E9) that is able to enhance the reaction with an effective molarity greater than 100 M (25). However, the affinities of the selected RNA molecules are almost five orders of magnitude lower than that of 1E9 (K_i=8 nM) (24). Therefore, it is likely that the lack of catalysis reflects an insufficient amount of binding energy available for the RNA to increase the effective concentrations of the two reactants with respect to one another or to stabilize the transition state for the cycloaddition reaction.

We are left to contemplate the prospects for isolating catalytic RNA molecules through transition state analog methodology. The highest affinities of RNA for small ligands are in the high nanomolar range (20, 21). The relatively sparse chemical features and lack of rotational freedom of nucleotides when compared to amino acids may prevent RNAs from tightly binding the transition state without also having an inhibitory affinity for the substrates or products of the reaction. Therefore, although RNA molecules have been isolated

that recognize small molecules with considerable affinity and specificity (17-21) the large, rigid bases may not be able to form binding pockets that complement the chemical topology of small ligands with the same accuracy of a protein binding pocket that is asked to "see" tiny differences in shape. Although we do not suggest that RNA is incapable of catalyzing reactions (particularly since overwhelming evidence to the contrary already exists), we predict that the difficulties in binding small transition state analogs with both higher affinity and specificity than substrates may limit the chemical transformations amenable to rate enhancement by nucleic acids.

The limited catalytic repertoire of nucleic acids might be enlarged through the use of several changes and additions to the SELEX procedure described in this report. The SELEX itself might have missed the highest affinity ligands (were they there) since column SELEX unavoidably presents the target molecules at high density. We might have tried counter-SELEX (21) with substrates or products to enrich those rare sequences that may strongly distinguish the transition state from the ground states of the reaction. In addition, there is compelling evidence that primitive RNA catalysts contained a larger number of bases than the four utilized in modern biological systems (31-33). Towards this end, many pyrimidines with additions at the 5-position are substrates for RNA polymerases and reverse transcriptases (34, 35, Bruce Eaton, personal communication), allowing nucleotides that provide additional chemical groups and more rotational degrees of freedom to be incorporated into the SELEX procedure. In an extension of this, RNA molecules with one of the

Diels-Alder substrates covalently attached to pyrimidines could be incubated with the second substrate followed by purification of sequences that have product covalently attached. This approach might allow direct selection for RNA-mediated catalysis of the cycloaddition reaction.

ACKNOWLEDGMENTS

We thank the members of our laboratories for numerous helpful comments and suggestions and Terri R. Gibson for preparation of the figures. This work was supported by National Institutes of Health Grants GM 28685 and GM 19963 to Larry Gold and GM 38273 to Donald Hilvert, by the Army Research Office and The Office of Naval Research Grant N00014-90-J-4089 to Donald Hilvert, and by a Damon Runyon-Walter Winchell Cancer Research Fund Fellowship, DRG-1139 to Kevin N. Morris. We are pleased to acknowledge the W. M. Keck Foundation and NeXagen for their generous support of RNA science on the Boulder campus.

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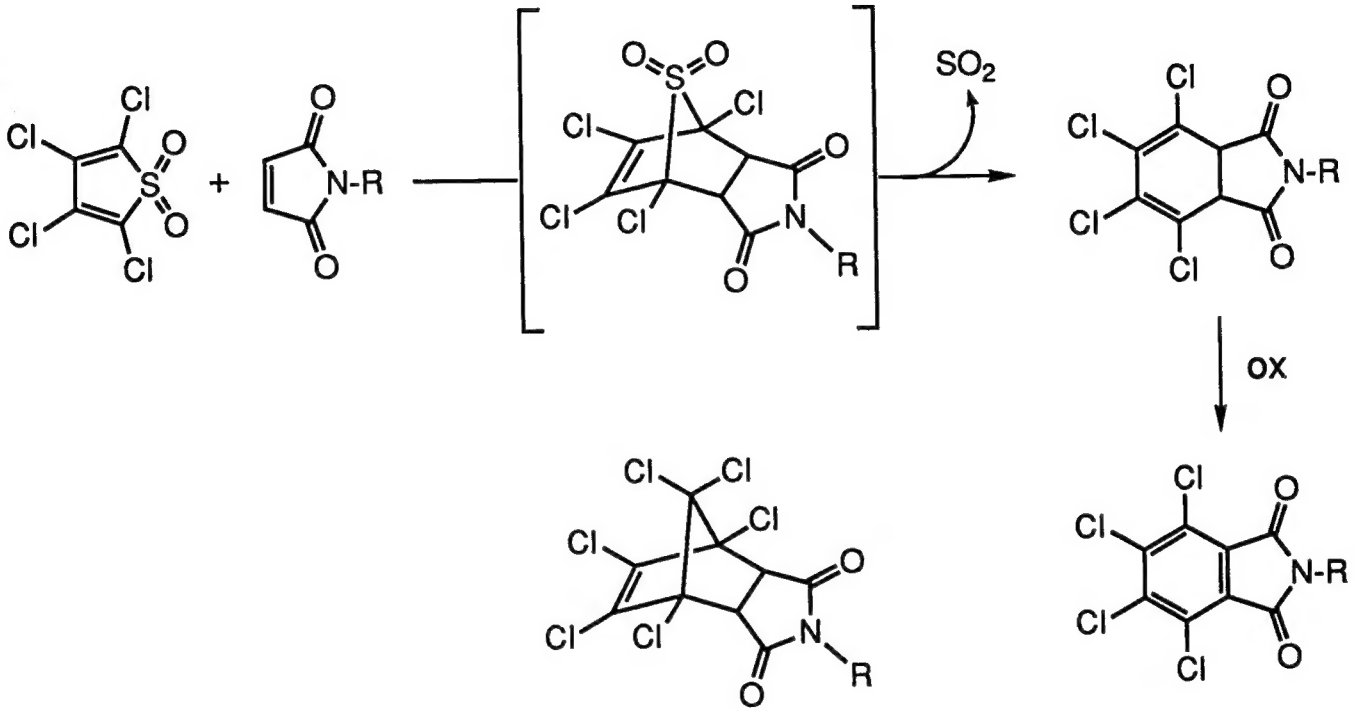
LEGENDS

Fig. 1. The Diels-Alder reaction between TCTD and NEM (25). The high energy state of the reaction is shown in brackets with the soluble form of the transition state analog (hexachloronorbornene derivative **1**) below.

Fig. 2. Elution profiles of the step gradient elutions of round 11, round 15-A and round 15-B as percent of the total amount of RNA applied to the column. The total percent of RNA eluted is also shown.

Fig. 3. (**A**) The consensus sequence identified in the RNA pools that recognize the hexachloronorbornene derivative **1** (Y=pyrimidine, R=purine). The percent conservation of each nucleotide after alignment of the sequences (26) is shown below. (**B**) The proposed secondary structure (27, 28) formed by a portion of the 5' fixed region (lower case letters) and the consensus sequence (upper case letters) at the 5' end of the random region. Base pairing interactions are shown as lines connecting the nucleotides (**C**) The proposed secondary structure formed by the partial consensus sequence in the RNA clone 15-B-9. Nucleotides that are conserved between this structure and that in (**A**) are denoted by an asterisk. An additional possible base pair that would make these two structures more similar is shown with a dotted line. (**D**) The 38-mer RNA that has the consensus sequence and secondary structure but failed to bind to the hexachloronorbornene derivative **1** column.

Fig. 4. The effect of deletions on the ability of the RNA clones 15-B-9 and 15-B-11 to bind to the hexachloronorbornene derivative **1** column. The percentage of a homogeneous population of each RNA that binds to the column and elutes with 50 mM soluble analog is given. The truncated RNA molecules are designated by the nucleotides that were deleted and are depicted graphically. The regions of fixed sequences and nucleotides that form the proposed conserved secondary structure are designated by the nucleotides in the primary sequence and are shaded with diagonal lines. The fixed sequence that contributes to the secondary structure in 15-B-11 is shown as cross-hatched.


Cl
Cl
Cl
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O
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N-R
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N
R
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SO2
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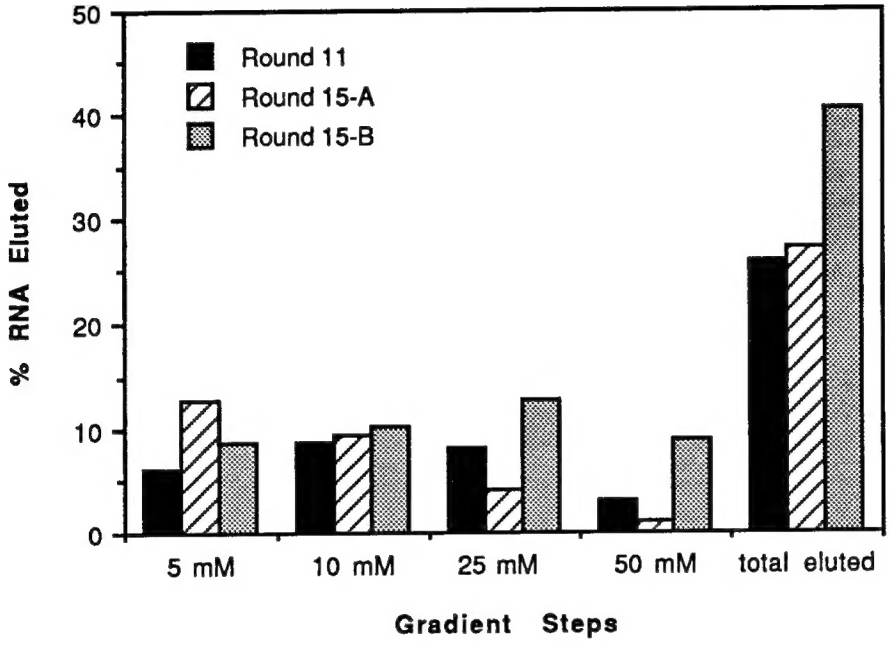
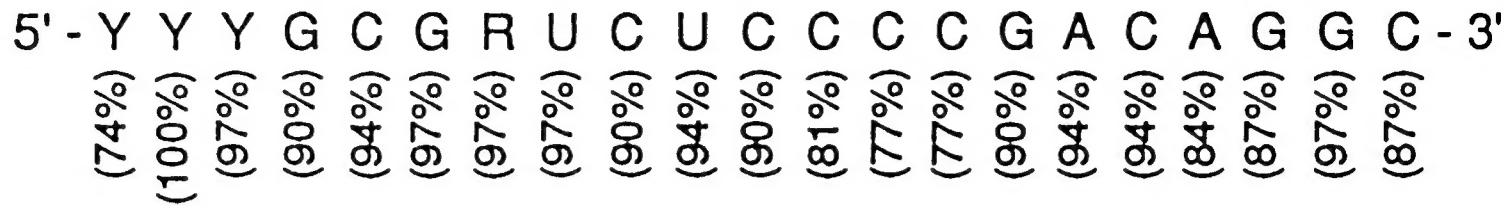
N-R
Cl
O

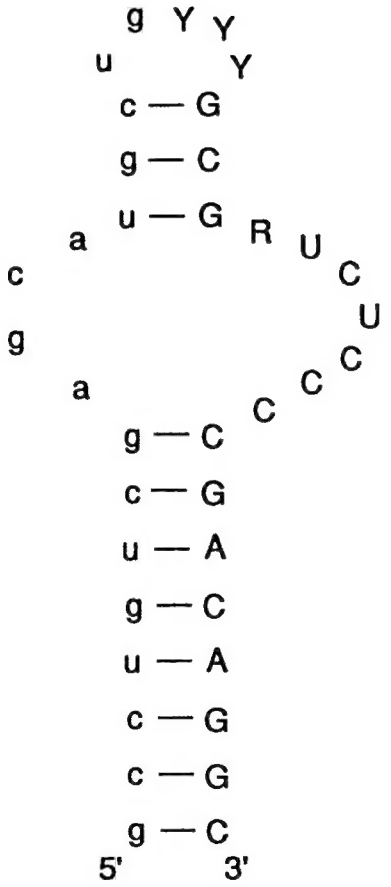
1 ($R=(CH_2)_5CO_2^-$)


50
40
30
20
10
0
% RNA Eluted
Round 11
Round 15-A
Round 15-B
5 mM
10 mM
25 mM
50 mM
total eluted
Gradient Steps

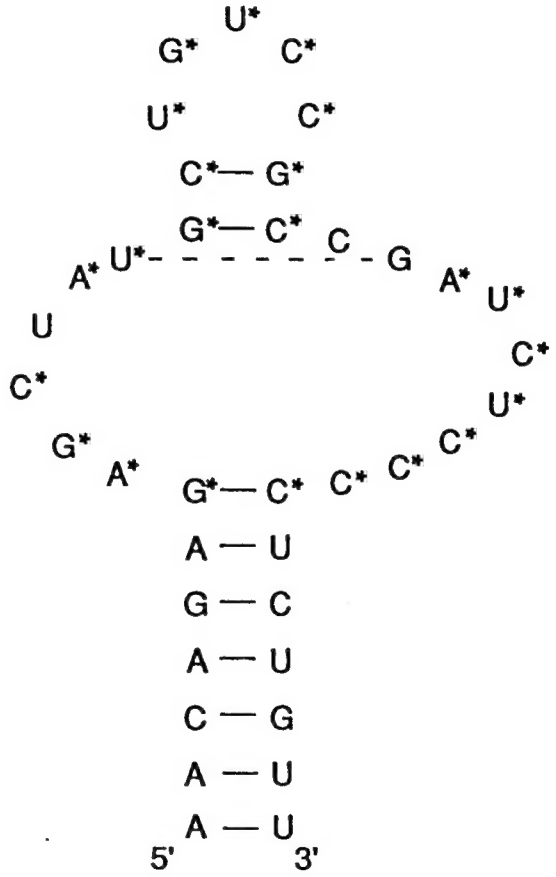
A


5' - Y Y Y G C G R U C U C C C C G A C A G G C - 3'
(74%) (100%) (97%) (90%) (94%) (97%) (97%) (97%) (90%) (94%) (90%) (81%) (77%) (77%) (90%) (94%) (94%) (84%) (87%) (97%) (87%)

B


5'
3'

C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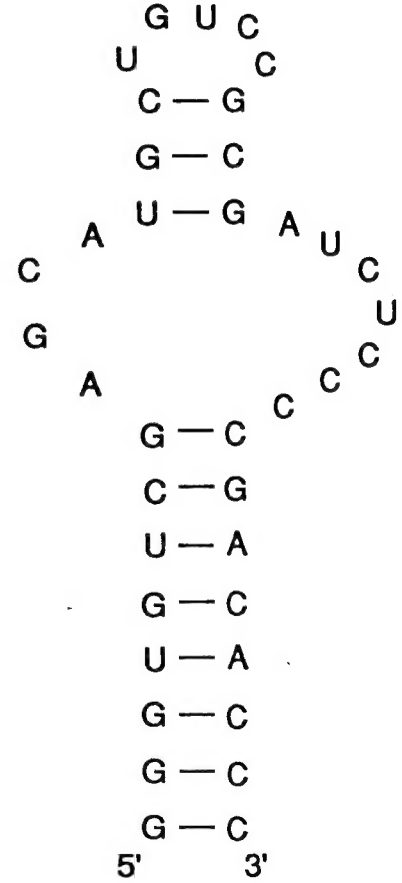
D


5'
3'

RNA	% RNA Eluting
15-B-9	34.0% ± 4.0%
15-B-9 (1-22)	3.6% ± 0.1%
15-B-9 (61-129)	<1%
15-B-9 (95-129)	45.0% ± 0.4%
15-B-11	28.0% ± 0.4%
15-B-11 (1-35)	<1%
15-B-11 (52-125)	4.7% ± 0.6%